



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C07K 14/74, C08B 37/00, C12N 5/08, 9/24, C12Q 1/48, 1/68, A61K 31/70, 38/46, 39/00, G01N 33/566	A1	(11) International Publication Number: <b>WO 96/17870</b> (43) International Publication Date: 13 June 1996 (13.06.96)
--	----	---

(21) International Application Number: PCT/US95/15913

(22) International Filing Date: 6 December 1995 (06.12.95)

(30) Priority Data:  
08/349,883 6 December 1994 (06.12.94) US(71)(72) Applicant and Inventor: ROTHENBERG, Barry, E.  
[US/US]; P.O. Box 997, Del Mar, CA 92014 (US).(74) Agents: WILSON, Barry, S. et al.; Campbell and Flores, Suite  
700, 4370 La Jolla Village Drive, San Diego, CA 92122  
(US).(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA,  
CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP,  
KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD,  
MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD,  
SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European  
patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU,  
MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM,  
GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE,  
LS, MW, SD, SZ, UG).

Published

*With international search report.*

(54) Title: IDENTIFICATION, PURIFICATION AND USES OF LECZYMES AND CARBOHYDRATE LIGANDS

## (57) Abstract

In various aspects of the present invention, carbohydrate ligands and peptide mimetopes of such ligands are provided which specifically bind to leczymses, and leczymses are provided which specifically bind to carbohydrate ligands and peptide mimetopes. Other aspects of the invention include methods for identifying, purifying and producing such carbohydrates, mimetopes and leczymses, and the use of such compositions in treating various diseases and otherwise modifying the functions of cells. Still other aspects of the invention involve diagnosis of various diseases involving carbohydrate ligands, peptide mimetopes of such ligands, and/or leczymses.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

IDENTIFICATION, PURIFICATION AND USES OF LECZYMES  
AND  
CARBOHYDRATE LIGANDS

BACKGROUND OF THE INVENTION

5

FIELD OF THE INVENTION

This invention relates generally to the field of glycobiology.

BACKGROUND INFORMATION

The major histocompatibility complex (MHC) codes for  
10 a variety of gene products, many of which play a central  
role in the body's defense against pathogenic organisms.  
Such molecules include the classical transplantation  
antigens and structurally related molecules, proteins for  
transport of foreign peptides within cells, serum  
15 complement proteins, the lymphokines tumor necrosis  $\alpha$  and  
tumor necrosis  $\beta$ , cytochromes and heat shock proteins.

The classical transplantation antigens, encoded for by  
genes in the MHC, are a highly polymorphic group of  
molecules that were originally discovered for their role in  
20 determining rejection of foreign transplanted cells and  
tissue. An extensive body of experimental work has since  
supported a role for the classical transplantation antigens  
in self-recognition. In the currently accepted paradigm,  
hereinafter the "old paradigm" transplantation antigens

s rve to present peptides derived from both self and foreign proteins, for recognition by cells of the immune system.

Two distinct groups of antigens, class I and class II  
5 antigens, are encoded by genes within the MHC. Class I  
antigens are expressed on virtually all nucleated cells in  
the body and play a role in the mediation of immune  
responses based on cytotoxic thymus-derived (T) lymphocyte  
mediated cell killing. Cytotoxic T lymphocytes play a role  
10 in killing of virus infected cells and tumor cells. The  
class I MHC molecule is composed of an approximately 45  
kiloDalton (kDa) heavy chain associated non-covalently with  
a 12 kDa protein known as  $\beta_2$  microglobulin ( $\beta_2$ M). The old  
paradigm characterizes class I antigens as presenting  
15 peptide fragments derived from both self and foreign  
proteins synthesized endogenously within the cell.

The class I molecules were discovered for their role  
in transplantation and were termed the "classical" class I  
molecules, to distinguish them from a later discovered  
20 group of class I molecules termed the "nonclassical" class  
I molecules. Genes encoding the nonclassical class I MHC  
molecules consist of the majority of genes so far  
identified in the MHC locus. Nonclassical class I MHC  
molecules are overall structurally related to the classical  
25 class I MHC transplantation antigens in having extensive  
sequence homology and a heavy chain noncovalently  
associated with  $\beta_2$ M. Nonclassical class I MHC molecules  
are, in general, less polymorphic than the classical class  
I MHC molecules and are more circumscribed in their tissue  
30 distribution. Several types of nonclassical class I

molecules are expressed principally in the gastrointestinal (GI) tract, raising questions regarding their function, if any in the immune system.

MHC class II antigens are expressed principally by  
5 specialized antigen presenting cells in the body. Such cells are limited to the antibody producing B lymphocyte as well as macrophages and dendritic cells distributed in various tissues of the body. The class II molecule on the cell-surface is composed of an  $\alpha$  chain of 33 kDa and a  $\beta$   
10 chain of 28 kDa associated noncovalently. Class II molecules as understood under the old paradigm function principally to present peptides derived from self or foreign proteins to a specialized class of T lymphocyte that supports the development of cytotoxic T lymphocytes,  
15 provides immunity to fungal infections and assists B lymphocytes in the generation of protective antibody responses to encapsulated bacterial infections. MHC class II antigens present peptide fragments derived from proteins taken up by cells from the surrounding environment, in  
20 contrast to classical class I molecules, which present peptides derived from endogenously synthesized proteins.

A variety of human autoimmune diseases have been shown to be associated more frequently in the population with individuals who inherit certain genes of the MHC. For many  
25 of these diseases, the association is localized to the region of the MHC encoding class II histocompatibility antigens. These diseases are not inherited by simple Mendelian segregation of MHC genes, since only one sibling of a set of identical twins may have the disease. This

feature suggests that other genetic factors or environmental factors have roles in the development of autoimmunity, with genes in the MHC playing a significant part of the process.

5       The old paradigm for MHC gene function provides several theories to explain a role for MHC genes in autoimmune disease. They include the inappropriate expression of class II MHC molecules in cells eliciting the autoimmune response or aberrant recognition of  
10 self-peptides by particular MHC gene products. Such theories, however, remain to be proven. In addition, the old paradigm fails to provide a useful hypothesis to explain the basis for an MHC-associated iron storage disease known as hemochromatosis. This disease is known  
15 from animal studies and from the genomic structure of several class I genes to involve an MHC encoded class I molecule since deletion of the  $\beta_2M$  gene in these animals results in the disease.

Disclosed herein is a new paradigm relating to  
20 carbohydrate-recognition molecules including at least some of class I, class II antigens and other related molecules, some of which are of the IgGSF. This paradigm provides that carbohydrate-recognition molecules have a central function to recognize and modify carbohydrate structures.  
25 Thus, there exists a need to develop new approaches to the treatment of MHC-associated and other diseases involving problems with carbohydrate-recognition, and in particular, a need to provide

composition, apparatus and methods for utilizing the new paradigm.

#### SUMMARY OF THE INVENTION

5        In various aspects of the present invention, carbohydrate ligands and peptide mimetopes of such ligands are provided which specifically bind to lecyzemes, and lecyzemes are provided which specifically bind to carbohydrate ligands and peptide mimetopes. Other  
10 aspects of the invention include methods for identifying, purifying and producing such carbohydrates, mimetopes and lecyzemes, and the use of such compositions in treating various diseases and otherwise modifying the functions of cells. Still other aspects of the invention involve  
15 diagnosis of various diseases involving carbohydrate ligands, peptide mimetopes of such ligands, and/or lecyzemes. Also provides are methods for modifying a cell to produce a carbohydrate ligand by introducing an expression vector encoding a lecyzyme into the cell,  
20 wherein the expression of the lecyzyme produces the carbohydrate ligand.

      In an exemplary embodiment, an immune response can be modulated by administering a carbohydrate ligand or a peptide mimetope to a patient. In another exemplary  
25 embodiment, hemochromatosis can be diagnosed by detecting a mutation in a class I MHC molecule that reduces its ability to associate with  $\beta_2$  microglobulin. In still another exemplary embodiment, the uptake of iron or other metal in a patient with abnormal absorption for

that metal can be modulated by administration of an appropriate carbohydrate.

#### DETAILED DESCRIPTION OF THE INVENTION

5       The present invention results from a profound new paradigm for the function of self-recognition molecules and other molecules in multicellular organisms, including mammals, which have a distinct population of phagocytic cells. The new paradigm holds that many types of  
10 self-recognition molecules heretofore known as peptide recognition and presentation structures have a more central function in the recognition and modification of carbohydrate-containing molecules. As used herein, "carbohydrate-containing molecules" means those molecules  
15 having at least one monosaccharide or sugar residue. Thus, self-recognition molecules and other molecules of the new paradigm have the ability to specifically bind a carbohydrate-containing molecule, and chemically modify it either by catalyzing further addition of carbohydrate  
20 or by catalyzing chemical modification of one or more of the existing carbohydrate residues. Therefore, the carbohydrate containing molecule is a substrate for the self-recognition and other molecules of the new paradigm. Additionally, after enzymatic modification, the  
25 self-recognition and other molecules can specifically bind with equivalent or greater affinity to the modified carbohydrate containing molecule than to the molecule originally recognized.



As used herein, "lecyzyme" means any cellular protein which can catalyze the chemical modification of a carbohydrate-containing substrate, resulting in a product with additional carbohydrate or chemically modified carbohydrate, and which then is capable of specifically recognizing the modified substrate. The term "lecyzyme" derives from the combination of having both lectin-binding and enzymatic activity in the same base molecule. Lecyzyme function is characteristic of many peptide recognition molecules that are well known in the art. Such molecules include at least some of class I and class II MHC encoded molecules, along with other members of the immunoglobulin gene superfamily (IgGSF) of molecules, some of which are not self-recognition molecules. Exemplary leczyzymes include those which catalyze phosphorylation, acetylation, carboxylation and/or sulfation of a carbohydrate-containing molecule. Leczyzymes can function within a cell, on the cell-surface, or can be secreted from a cell.

20

A leczyzyme can exhibit enzymatic activity and carbohydrate binding activity in the same isoform of the molecule or these activities can reside separately in different isoforms of the molecule. For example, differential RNA splicing of a leczyzyme can result in an enzymatically active isoform of the leczyzyme which contains a signal(s) directing the leczyzyme to sites in the cell normally associated with glycosylation, such as the endoplasmic reticulum or the golgi complex. Differential RNA splicing can also result in an isoform of the leczyzyme that exhibits carbohydrate recognition

30

capability and contains a signal(s) directing the receptor to the cell-surface or to export from the cell. Alternatively, a leczyne expressed either in the cell or on the cell-surface can contain both enzymatic activity  
5 as well as carbohydrate recognition capability in the same molecule.

In class I or class II molecules, and possibly in other leczyne, leczyne function can be resident in the  
10 groove formed at the top of MHC encoded molecule. The old paradigm characterizes the groove as a peptide-binding groove, but the new paradigm provides that the groove functions principally to recognize a carbohydrate structure and its subsequent form after  
15 modification by the leczyne.

Compositions of substantially purified carbohydrate ligands can be isolated which specifically bind to a leczyne. As used herein, the term "carbohydrate ligand" means a carbohydrate-containing molecule where the  
20 carbohydrate portion is a part of the ligand that is recognized by the leczyne. A carbohydrate ligand is composed of one or more sugar residues that are linked in either straight chain or branched chain configuration. Therefore, a carbohydrate ligand includes a  
25 monosaccharide, disaccharide, trisaccharide, oligosaccharide or polysaccharide.

Carbohydrate ligands composed of multiple carbohydrate residues can vary in the type and location of the linkage between each residue. Carbohydrate

residues useful for producing a carbohydrate ligand include, for example, glucose, galactose, fucose, mannose and sialic acid. Carbohydrate residues of a ligand also can be acetylated, phosphorylated and/or sulfated by  
5 chemical processes well known in the art. A carbohydrate ligand also can be chemically bonded to other molecules such as a lipid, glycolipid, protein, glycoprotein, proteoglycan, glucosaminoglycan or an organic molecule. Such additional molecules can provide the carbohydrate  
10 ligand with features such as increased binding to the leczyne or increased stability *in vivo*.

A carbohydrate ligand can be multivalent in nature by having more than one carbohydrate ligand attached to a backbone structure. The backbone structure can be a  
15 natural protein such as a serum albumin or can be a synthetic molecule such as a synthetic peptide. Approaches to link multiple carbohydrate ligands to a backbone structure are known in the art and include, for example, biotin-avidin linkage (Rothenberg et al., Proc.  
20 Natl. Acad. Sci. (USA) 90:11939-11943 (1993), which is incorporated herein by reference).

Application of the new paradigm yields new methods to treat disease states resulting from leczymses. Such disease states may include, for example, autoimmunity,  
25 hemochromatosis, inflammation, transplantation rejection, and infections. In many of the above disease states, disease may result in part from aberrant recognition of carbohydrate structures by lymphocytes. Thus, the administration of a carbohydrate ligand that can bind to

the aberrant recognition molecule of an individual provides a means to disrupt the aberrant recognition mediating the disease.

A variety of leczyms exist that differ in their  
5 ability to modify particular types of molecules. This difference results in part from differences in the specificity of the lectin binding site that leczyms have for their substrate. Thus, a part of the leczyms structure is a recognition site for the substrate. The  
10 catalytic site of a leczyms can be the same site as the substrate recognition site or can be a site different from the substrate recognition site. After modification of the substrate, the leczyms can exhibit similar or greater binding affinity for the modified substrate over  
15 the original substrate due to coordinate binding by both the substrate recognition site and the catalytic site of the leczyms or by multivalency of the ligand.

Leczyms provided herein include a broad group of structurally related molecules, many of which are  
20 contained within the IgGSF. The IgGSF series of genes share an evolutionary homology (i.e., common ancestor) but are not necessarily functionally related, genetically linked or coordinately regulated. The products of the IgGSF have been defined by the presence of one or more  
25 regions homologous to the basic structural unit of immunoglobulin (Ig), known as the Ig homology unit. These units are characterized by a primary amino acid sequence of about 70-100 residues in length and include an essentially invariant disulfide bridge spanning 50-70

residues in length and several other relatively conserved residues that maintain a tertiary structure known as the Ig fold (for review see Hunkapiller and Hood, Adv. Immunol., 44:1-63, (1989)).

5       The genes of the IgGSF encode many molecules with known immunological function, such as the immunoglobulins, T lymphocyte receptors, classical and nonclassical MHC molecules, various T lymphocyte and B lymphocyte cell-surface molecules or  $\beta_2$ M. In addition,  
10 the IgGSF encodes several cell-surface molecules known to function as receptors for cell-cell adhesion. Such adhesion molecules include, for example, the neural cell adhesion molecule carcinoembryonic antigen. Of course, those IgGSF molecules which either do not modify the  
15 carbohydrate portion of the carbohydrate ligand, or which do not recognize the modified product are not lecymses.

Leczymes of the IgGSF include those encoded by genes located within the MHC region. In humans, the MHC is in a continuous stretch of DNA located on the short arm of  
20 chromosome 6. The entire MHC in humans is called the HLA complex. In mice, the MHC is located on chromosome 17 and contains the H-2, Q, T and M complexes. As used herein, the term "MHC-derived gene product" means any molecule that contains at least one polypeptide encoded  
25 for by a gene located within the MHC. Leczymes that are MHC-derived gene products include class I and class II molecules. Class I and class II molecules that are leczymses in humans are encoded by genes within the HLA-D region such as HLA-DP, HLA-DN, HLA-DM, HLA-DO, HLA-DQ or

HLA-DR, or the various alleles of HLA-A, HLA-B and HLA-C loci, or the HLA-X, HLA-E, HLA-J, HLA-H, HLA-G and HLA-F genes.

Leczymes that are class I MHC molecules contain an  
5 approximately 45 kDa polymorphic heavy chain or  $\alpha$  chain  
associated noncovalently with a small nonpolymorphic  
protein called  $\beta_2M$ . The heavy chain is an MHC-encoded  
gene product located in or near the A, B or C regions of  
the human HLA complex and within or near the K or D/L  
10 regions of the mouse H-2 complex. Although  $\beta_2M$  is encoded  
by a gene located outside the MHC and on a different  
chromosome, the heavy chain of the class I molecule is  
encoded by a gene located within the MHC, thereby  
including a class I molecule within the definition of an  
15 MHC-encoded gene product.

Leczymes that are class II MHC molecules include  
those MHC-derived gene products composed of a 34 kDa  $\alpha$   
chain associated noncovalently with a 28 kDa  $\beta$  chain. An  
additional chain called the invariant chain is  
20 transiently associated with the class II heterodimer  
during transport to the plasma membrane of the cell.

Leczymes can be expressed on the cell-surface by  
virtue of having a transmembrane region and cytoplasmic  
tail, as in the case of the classical transplantation  
25 antigens. Leczymes also can be linked to the  
cell-surface in a manner similar to some nonclassical  
class I molecules. For example, many of the nonclassical  
class I Qa and Tla molecules are linked to the

cell-surface by a phosphatidylinositol (PI) linkage, and the product of the Q10 gene appears to be secreted (Devlin et al., EMBO J. 4:369-374 (1985)). The majority of Qa and Tla antigens lack the classical class I  
5 cytoplasmic exons including the phosphorylation site in exon seven (Thor et al., J. Immunol., 151:211-224 (1993)), although the transmembrane domain and the seventh exon is present in Q1 and Q2 gene products.

The MHC class I heavy chain is organized into three  
10 external domains ( $\alpha 1$ ,  $\alpha 2$  and  $\alpha 3$ ), each containing about 90 amino acids each, a transmembrane domain of about 40 amino acids and a cytoplasmic anchor segment of about 30 amino acids.  $\beta_2M$  is similar in size and in organization to the external  $\alpha 3$  domain of the heavy chain. X-ray  
15 crystallographic analysis of the extracellular portion of the MHC class I molecule shows that the  $\alpha 1$  and  $\alpha 2$  domains interact and are most external to the cell membrane while the  $\alpha 3$  and  $\beta_2M$  domains interact and are more proximal to the cell membrane. The interacting  $\alpha 1$  and  $\alpha 2$  domains  
20 form a platform containing a deep groove or cleft located on the top surface of the molecule.

The old paradigm for the function of the classical class I MHC molecule interprets the groove at the top of  
25 the molecule as a peptide binding site. The site is sufficiently large enough to bind a peptide of about 8-20 residues in length and present both self and foreign-derived peptides for recognition by certain T lymphoid cells. Extensive research has shown that the  
30 MHC classical class I molecule can bind peptide of about

the length of the groove. In addition, the x-ray crystallographic analysis of a classical class I molecule purified from a cell indicated that a peptide was resident in the groove. However, as described above, the  
5 new paradigm in the present invention provides that the peptide binding groove of the classical class I molecule MHC is also suited for binding a carbohydrate ligand.

Leczymes that are a class II MHC molecule include those that share significant structural features with a  
10 class I molecule. The class II molecule is a membrane bound glycoprotein that contains external domains, a transmembrane segment, and a cytoplasmic anchor segment. The  $\alpha$  chain contains two external domains labeled  $\alpha 1$  and  $\alpha 2$  and the  $\beta$  chain contains two external domains  $\beta 1$  and  
15  $\beta 2$  domain. X-ray crystallography shows that the  $\alpha 2$  and  $\beta 2$  domains interact as a membrane proximal structure analogous to the  $\alpha 3$  domain and  $\beta_2 M$  domain interaction of the class I molecule. The  $\alpha 2$  and  $\beta 2$  domains of a class II molecule that together form a cleft at the top of the  
20 molecule that is very similar to the cleft formed by the  $\alpha 2$  and  $\alpha 3$  domains of a class I heavy chain. Extensive evidence indicates that the groove in the class II molecule can bind and present both self and foreign peptides for recognition by T lymphoid cells. Peptides  
25 have been isolated from the class II molecule that are from 13-18 amino acids in length, slightly longer than the octomeric or nonomeric peptides commonly isolated from MHC classical class I molecules. As discussed above, the new paradigm of the present invention provides  
30 that the peptide binding groove in the class II MHC



molecule, like the groove in the classical class I MHC molecule, is also suited for binding a carbohydrate ligand.

Leczymes may also be encoded by nonclassical class I  
5 genes. In the mouse, genes encoding leczyms are located  
in the MHC regions Q, T and M downstream of the classical  
histocompatibility antigens. There are similar regions  
in humans coding for known nonclassical class I molecules  
such as HLA-F and HLA-G. The nonclassical class I genes  
10 are overall less polymorphic than the classical class I  
genes and show different patterns of expression. The Q,  
T and M complex genes of mice consist of approximately 45  
genes, coding for non-polymorphic differentiation  
antigens with limited tissue distribution.

15 Known leczyms which are nonclassical class I MHC  
molecules exhibit limited tissue distribution in  
comparison with leczyms that are classical class I MHC  
molecules. For example, the Qa and Tla antigens, the  
products of the Q and T genes, are expressed on  
20 subpopulations of lymphocytes (for review, see Flaherty  
et al. Critical Reviews in Immunology, 10:131-175  
(1990)). Previously, no convincing function had been  
assigned to the products of the nonclassical class I  
genes, although they have been suggested as possible  
25 restriction elements for  $\gamma\delta$  T cells (Hershberg et al.  
Proc. Nat. Acad. Sci (USA), 87:9727-31 (1993)). The Qa  
and Tla antigens have also been reported to be expressed  
on intestinal epithelium (Wu et al, J. Exp. Med.,  
174:213-218 (1991); Hershberg et al., Proc. Natl. Acad.

Sci. (USA) 87:9727-97231 (1990); Wang et al.,  
Immunogenet., 38:370-372 (1993)) where their function was  
unknown. The new paradigm of the present invention  
provides that at least some of these nonclassical class I  
5 molecules are leczyms.

The nonclassical class I molecule Q2, produced by a  
gene within the mouse MHC, is an example of a leczyms  
that is involved in iron transport (see Example I). In  
this instance a mucin or other carbohydrate containing  
10 molecule acts as an iron chelator, and in turn also  
contains the ligand for leczyms expressed from the Q2  
gene. The gene for Q2 is located in a head to head  
relationship with another gene most likely encoding a  
mucin. Both genes share a single promoter region,  
15 located between the genes, the promoter being analogous  
in structure to the  $\beta$ -globin promoter involved in iron  
metabolism. The coordinated regulation of these two genes  
can be readily understood in view of the receptor/ligand  
and receptor/substrate interactions defined as leczyms  
20 function in the new paradigm. Interestingly, the Q2 gene  
is distinguished from other nonclassical class I genes in  
being highly polymorphic with Q2 molecules of different  
strains of mice differing significantly in amino acid  
sequence. Despite these differences, the Q2 molecules  
25 from separate strains of mice all function as a receptor  
for their co-regulated gene product since, as a leczyms,  
Q2 can enzymatically modify its ligand/substrate in  
accordance with the lectin recognition and enzymatic  
function of each Q2 gene product and can recognize the  
30 resulting product. Thus, the combined

enzymatic/recognition capability of a leczyne as defined in the new paradigm maintains receptor/ligand relationships in the face of extensive genetic polymorphism.

5        Leczymes exist with a variety of enzymatic activities. For example, a leczyne can have a glycosyl transferase enzymatic activity that results in the catalytic transfer of a glycosyl group (mono or carbohydrate) from a glycosylnucleotide to an acceptor  
10 molecule such as a protein, carbohydrate or lipid. However, not all glycosyl transferases are leczyms.

There is currently only one glycosyl transferase ( $\beta$ 1,4-galactosyltransferase) that is previously known to be expressed in both the cytoplasm and on the cell (for  
15 a review see Shur, Curr. Opin. in Cell Biol., 5:854-863 (1993)). This enzyme has both carbohydrate recognition capability and carbohydrate catalytic activity and has been implicated in a variety of cell-cell and cell-matrix interactions. One hallmark of the cell-surface expressed  
20 form of  $\beta$ 1,4-galactosyltransferase is that it no longer retains binding activity for the product it generates after enzymatic modification (Miller et al., Nature, 357:590-593 (1992)). Thus, this particular transferase is not a leczyne because it fails to exhibit recognition  
25 for its enzymatic product.

A leczyne of the IgGSF can be encoded by a gene located outside the MHC. For example, CD-1 is a product of the IgGSF gene that is related in structure to the

class I MHC molecule, but the CD-1 heavy chain is encoded by a gene outside the MHC. The T-6 CD-1 molecule is expressed by a specialized antigen presenting cell in the skin (Langerhan's cell) and can be internalized into the  
5 cell along with MHC class II antigen, indicating an immunological function for T-6.

A composition can be prepared which comprises a substantially purified carbohydrate ligand that is specifically bound by a leczyne. As used herein, the  
10 term "substantially purified" means a carbohydrate ligand that is relatively free from other contaminating molecules such as lipids, proteins, nucleic acids, carbohydrates or other molecules which are not normally associated with the carbohydrate ligand. Also as used  
15 herein, the term "specifically bound" means having sufficient complementarity to achieve a concentration of unbound substrate in the presence of sufficient leczyne of at least  $10^{-4}$  Molar, and preferably  $10^{-6}$  Molar or greater. A substantially purified carbohydrate ligand  
20 can be obtained, for example, using well known biochemical methods of purification of a carbohydrate source or by chemical or enzymatic synthesis.

A carbohydrate ligand of the present invention can include known carbohydrate containing molecules such as  
25 glycoproteins, proteoglycans, glycolipids or mucopolysaccharides that have N-linked or O-linked forms of glycosylation. Proteoglycans include, for example, mucins and those proteoglycans glycosylated with hyaluronate, chondroitin sulfate, heparin, heparin

sulfate or dermatin sulfate. Glycolipids include, for example, acylglycerols, sphingoids and ceramides.

A sample containing a substantially purified carbohydrate ligand can be obtained from a variety of sources such as from fluids, tissues or cells. These sources can be from any plant species or any animal such as a mammal or any organism. A source of carbohydrate ligand can also include a cell that has been modified by introducing into the cell an expression vector that encodes a leczyne or a protein that when expressed contains a carbohydrate ligand.

A sample containing a carbohydrate ligand can be obtained from a chemically produced library of carbohydrates. Such libraries can be made by mixing carbohydrates from natural sources and from enzymatically-produced sources. In addition, individual carbohydrates from the library can be tagged with a detectable label such as a fluorescent label to assist in structural determination of the carbohydrate ligand.

A sample containing a carbohydrate ligand can be processed to further purify the ligand by methods well known in the art. Such methods include, for example, purification of glycoconjugates, labeling of glycoconjugates by chemical or metabolic means, release of carbohydrates from glycoconjugates and characterization of the structure of the released carbohydrate (see, for example, Ausabal et al, In Current Protocols in Molecular Biology Vol. 2, chapter 17, (Green

Publishing Associates and Wiley Interscience, New York, 1994); Fukuda and Kobata, Glycobiology: A practical Approach, (IRL Press, New York, 1993), both of which are incorporated herein by reference). In addition, these  
5 methods are useful for structural characterization, including sequencing of the carbohydrate ligand. Elucidation of the structure of a carbohydrate ligand purified from a tissue or a cell can enable future production of the ligand by direct chemical synthesis or  
10 enzymatic synthesis or purification from a natural source.

A carbohydrate ligand that can bind to a leczyne can be identified by several methods. In one method, a sample containing a carbohydrate ligand is contacted with  
15 a leczyne suspected of binding to the ligand under suitable conditions to allow specific binding of the ligand to the leczyne. Suitable conditions include, for example, an appropriate buffer concentration and pH and time and temperature that permits binding of the  
20 particular leczyne and the carbohydrate ligand. After a suitable reaction period, the amount of carbohydrate ligand bound to the leczyne can be determined, for example, by attaching a detectable moiety such as a radionuclide or a fluorescent label to the carbohydrate  
25 ligand and measuring the amount of label that is associated with the leczyne after any unbound carbohydrate ligand has been removed from the ligand-leczyne complex.

As used herein, "detectable label" means a molecule whose presence can be detected due to a physical, chemical or biological characteristic of the molecule. Detectable labels include, for example, radioisotopes, fluorescent molecules, enzyme/substrate systems, or visually detectable molecules. Methods for detectably labeling a carbohydrate molecule are well known in the art, and include, for example, reduction with NaB(<sup>3</sup>H), or synthesis with radiolabelled carbohydrates (see, for example, Varki, *supra*, 1994 and Rothenberg et al., Proc. Natl. Acad. Sci. (USA), 90:11939-11943 (1993), both of which are incorporated herein by reference, and Fukuda and Kobata, *supra* 1993). In addition, kits for the preparation of a labelled carbohydrate molecule are readily available from commercial sources such as Oxford GlycoSystems (Rosedale, NY).

Methods to remove unbound labelled ligand from the ligand-enzyme complex may depend, for example, on attaching the enzyme to a solid support. Solid supports useful in the present invention and methods to attach proteins to such supports are well known in the art (see for example Harlow and Lane, Antibodies: A laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988), which is incorporated herein by reference). Such solid supports include, for example, Sepharose, agarose or polystyrene.

After a suitable reaction period and after any unbound label has been removed from the support by, for example, washing, the amount of label attached to the

solid support provides a direct measurement of the amount of carbohydrate ligand bound to the leczyne on the support. Alternatively, the amount of labelled carbohydrate ligand bound to the support can be  
5 indirectly determined after the reaction period by measuring the amount of unbound label and subtracting this from the amount of label added at the start of the reaction.

To accurately determine the amount of labelled  
10 ligand that binds specifically to the leczyne, a control reaction can be performed where all conditions are the same as in the binding reaction between the labelled ligand and leczyne except that the leczyne is not included in the control reaction or the leczyne is  
15 replaced by an irrelevant protein such as a serum albumin. The control reaction determines the amount of binding of the labelled carbohydrate ligand that occurs nonspecifically such as the amount of labelled ligand that binds to the solid support rather than to the  
20 leczyne on the solid support. Thus, it is necessary to subtract the nonspecific binding value obtained from the control reaction from the binding value obtained from the reaction that included both the labelled carbohydrate ligand and the leczyne to determine the amount of ligand  
25 that specifically bound the leczyne under the conditions tested.

An advantage of using a solid support is that the labelled ligand can be added in excess relative to the leczyne, making it possible to identify lower levels of



binding affinity between the carbohydrate ligand and the  
leczyme. Methods such as Scatchard analysis are well  
known in the art for determining the binding affinity  
between two molecules, both of which can be in solution  
5 or one of which can be attached to a solid support.  
Equilibrium dialysis is an example of a method where the  
binding of a ligand to leczyme can be determined when  
both molecules are in solution.

Methods to measure the binding of a labelled  
10 carbohydrate ligand to a leczyme also can be performed  
when the leczyme is associated with a cell. In a manner  
analogous to the use of solid supports, cells that  
express the leczyme on the cell-surface can bind the  
labelled carbohydrate ligand and, after a suitable  
15 reaction period, the cells can be separated from the  
unbound ligand by methods well known in the art such as  
by centrifugation or filtration. Cells that express a  
leczyme in the cytoplasm can also be used to detect  
binding of a carbohydrate ligand to the leczyme provided  
20 the cell membrane has been sufficiently permeabilized to  
allow access of the carbohydrate ligand to the leczyme in  
the cell. Methods that use cells in binding assays such  
as antigen-antibody binding assays are well known in the  
art (see, for example, Harlow and Lane *supra*, 1988) and  
25 are generally applicable to binding assays between a  
carbohydrate ligand and a leczyme.

A leczyme-expressing cell can be a cell that  
naturally expresses the leczyme, such as a lymphocyte  
that expresses a class I or class II MHC encoded leczyme.

A leczyne-expressing cell can also be a cell that expresses the leczyne as a result of introducing an expression vector encoding the leczyne into the cell. Leczyne-expressing cells can be obtained from in vivo  
5 sources by methods well known in the art such as mechanical disruption of tissue or digestion of tissue by enzymes to release cells from their surrounding matrix (see for example, Freshney Culture of Animal Cells (Alan R. Liss, New York, 1993), which is incorporated herein by  
10 reference). A leczyne-expressing cell can be a cell line that is available from public cell repositories such as from the American Type Culture Collection.

It is well known in the art that the binding between two molecules can be performed when either of the two  
15 molecules contains a detectable label. Thus, the identification of a detectably labelled carbohydrate ligand that binds to a leczyne attached to a solid support or a cell also can be performed if the leczyne contains the detectable label and the carbohydrate ligand  
20 is attached to a solid support or expressed by a cell. A leczyne can be detectably labelled using methods for labeling a protein, which are well know in the art and include, for example, biotinylation or incorporation of radioisotopic labelled precursors. A carbohydrate  
25 ligand-expressing cell can be a cell obtained from tissues or organs or can be a cell line such as a cell line available from a public repository.

Methods for attaching a carbohydrate ligand to a solid support depend on the chemical nature of the

ligand. Thus, attachment can be accomplished through the carbohydrate moiety or other molecule bonded to the carbohydrate ligand attachment via chemistry suitable for attaching carbohydrate, peptide or lipid structures to a solid support. Methods to attach carbohydrates, proteins or lipids to various types of solid supports are well known in the art.

The binding of a carbohydrate ligand to a leczyne can be determined without the need for a detectable label by measuring a physical characteristic of the either the ligand or the leczyne such as absorption of ultraviolet radiation. Such methods for quantitating a protein or carbohydrate by physical characteristics are well known in the art. The ability to follow a physical characteristic of the ligand or leczyne can be applied to binding assays that use a solid support or an expressing cell or when both molecules are in solution. The binding of a carbohydrate ligand to a leczyne also can be evaluated if the ligand is a substrate for the enzymatic activity of the leczyne. In this case, binding can be measured by following substrate conversion kinetics measured, for example, by the Michealis-Menten equation (Devlin, Textbook of Biochemistry (Wiley-Liss Inc. New York, 1992), which is incorporated herein by reference).

Methods for identifying a carbohydrate ligand that binds a leczyne can be performed using a single purified carbohydrate ligand or a limited number of carbohydrate ligands, which can be purified by conventional procedures as described above or can be purified by binding to a

binding reagent. As used herein, " binding reagent" means a chemical or biological molecule that can specifically bind to a carbohydrate ligand. For example, a leczyne that binds to a carbohydrate ligand is a  
5 reagent that can be used to purify that ligand. Also, an antibody can be a reagent if it can react specifically with the carbohydrate, protein or lipid portion of a carbohydrate ligand.

A purified carbohydrate ligand can also be  
10 detectably labelled by methods disclosed herein. A carbohydrate ligand that is not purified, such as one that is in a sample containing other molecules, can be used in a binding assay provided it is attached to a solid support or is expressed by a cell and binding is  
15 determined by detecting binding of a leczyne. In this case, if the non-purified carbohydrate ligand can bind the leczyne, the sample containing the ligand can be subjected to purification and subsequent binding assays to obtain the carbohydrate ligand in a purified state.

20

Purified leczyms can be obtained from cells by classical methods for protein or glycoprotein purification such as methods known in the art for purifying class I or class II molecules. Leczyms also  
25 can be obtained from cells that have been modified by molecular biological techniques to enable expression of a leczyne. A gene encoding a leczyne can be cloned into an expression vector and then introduced into a host cell. Vectors are well known in the art and include, for  
30 example, cloning vectors and expression vectors, as well

as plasmids or viral vectors (see, for example, Goedell, Methods in Enzymology, vol. 185 (Academic Press, New York, 1990), which is incorporated herein by reference). A baculovirus vector is an example of a vector that can  
5 be used to express a leczyne in insect cells and result in expression of new carbohydrate ligands on the cell.

A vector comprising a nucleic acid molecule encoding a leczyne also can contain a promoter or enhancer element, which can be constitutive or inducible and, if  
10 desired, can be tissue specific. Host cells also are known in the art and an appropriate host cell can be selected for the particular vector to be used. For example, a baculovirus transfer vector can be used with baculovirus DNA to infect insect cell lines such as SF21  
15 cells. Cloning of such transformed cells to produce a stable cell line can provide a source of the expressed leczyne or can provide a source of carbohydrate ligand modified by the expressed leczyne.

The gene encoding a leczyne can be expressed as a  
20 fusion protein to assist in purification or in further downstream processing of the leczyne. For example, the leczyne can be produced as a chimeric protein fused to the CH2 or CH3 domain that constitutes the Fc binding region of an immunoglobulin molecule, as was performed  
25 previously for expressing the CD22 $\beta$  lectin (Stamenkovic et al. Cell, 66:1133-1144 (1991)). The use of Protein A from *Staphylococcus aureus* bound to a solid support, which is readily available from commercial sources, can be used to purify the Fc containing chimeric leczyne. In

addition, the solid support containing the chimeric leczyne can be used directly to evaluate binding of a carbohydrate ligand.

The methods that have been described above for  
5 identifying a carbohydrate ligand that binds to a leczyne  
can also be used to identify a leczyne that binds to a  
carbohydrate ligand. For example, a sample containing a  
leczyne is contacted with a carbohydrate ligand suspected  
of binding to the leczyne under suitable conditions to  
10 allow specific binding of the ligand to the leczyne.  
Leczymes to be identified for binding include, for  
example, a purified leczyne or a leczyne contained within  
a complex mixture such as a mixture of proteins expressed  
from a cDNA expression library. Methods to produce a  
15 cDNA expression library are well known in the art (see,  
for example, Sambrook et al, Molecular Cloning: A  
laboratory manual (Cold Spring Harbor Laboratory Press  
1989), which is incorporated herein by reference).

A carbohydrate ligand can also be purified by an  
20 affinity column and related technologies. For example, a  
sample containing the carbohydrate ligand can be  
contacted with the binding reagent under suitable  
conditions to allow formation of a ligand-binding reagent  
complex. Suitable conditions includes, for example, an  
25 appropriate buffer concentration and pH and time and  
temperature that permits binding of the carbohydrate  
ligand to the binding reagent. The ligand-binding  
reagent complex is then separated from the rest of the

sample by a separation method such as by washing, and the ligand is dissociated from the complex.

As another example, a leczyne that binds to a carbohydrate ligand may comprise a binding reagent used  
5 to purify that ligand. An antibody can also comprise a binding reagent if it can react specifically with the carbohydrate, protein or lipid portion of a carbohydrate ligand.

Purification of the carbohydrate ligand can be  
10 accomplished if the binding reagent is attached to a solid support such as agarose, Sepharose or plastic. Methods for coupling a protein or a carbohydrate to a solid support, disclosed above for detecting the binding of a carbohydrate ligand to a leczyne, also are useful  
15 for attaching a binding reagent to a solid support.

Methods to dissociate a carbohydrate ligand from a ligand-binding reagent complex can depend on the nature of the binding reagent. For example, if the binding reagent is a leczyne, then a method for dissociating the  
20 complex can involve competitive inhibition of the complex with a carbohydrate structure that has binding affinity for the same site in the leczyne that binds the carbohydrate ligand. Other well known treatments that are useful for dissociating a carbohydrate ligand from a  
25 binding reagent include, for example, extremes in pH, high salt concentration or chaotropic agents (see, for example, Harlow and Lane, *supra*, 1988), which is incorporated herein by reference and Varki, *supra*, 1994).

Carbohydrate ligands purified by the above disclosed methods are suitable for structural analysis as described above, in order to enable future production of the ligand by chemical or enzymatic synthesis.

5       An antibody that specifically binds to an oligosaccharide or polysaccharide-type carbohydrate ligand can be produced to the carbohydrate, or to a protein moiety or a lipid moiety if such moieties are bonded to the ligand. An antibody specific for the  
10 peptide backbone of carbohydrate ligand such as the peptide backbone of a mucin can be useful for purifying a source of mucin from different cells or from different individuals, since the peptide backbone can be more conserved between peptide containing carbohydrates than  
15 the carbohydrate portions of these molecules. Methods for producing antibodies such as polyclonal antibodies, monoclonal antibodies, antibody fragments or the like, that are specific for protein, carbohydrate or lipid are well known in the art (see, for example, Harlow and Lane  
20 *supra*, 1988).

A leczyne can be purified using methods whereby the leczyne specifically binds to a carbohydrate ligand. In these methods, a sample containing the leczyne is contacted with a carbohydrate ligand under suitable  
25 conditions to allow formation of a ligand-leczyne complex. Suitable conditions includes, for example, an appropriate buffer concentration and pH and time and temperature that permits binding of the leczyne and the carbohydrate. The ligand-binding reagent complex is then



separated from the rest of the sample by a method such as by washing, and the leczyne is dissociated from the complex.

Purification of the leczyne can be accomplished if  
5 the carbohydrate ligand is attached to a solid support such as agarose, Sepharose or plastic. Methods for coupling a carbohydrate ligand to a solid support, such as those disclosed above for detecting the binding of a carbohydrate ligand to a leczyne, are useful for  
10 attaching a carbohydrate ligand to a solid support. Methods for dissociating the leczyne from the ligand-leczyne complex can utilize the methods disclosed herein for dissociating a carbohydrate ligand from a ligand-leczyne complex.

15 A carbohydrate ligand that modifies the function of a leczyne-expressing cell can be identified by contacting a sample containing a carbohydrate ligand with the cell under suitable conditions, which allow specific binding of the ligand to the leczyne on the cell. After a  
20 suitable period of time to allow for binding of the ligand to the leczyne, the cells are evaluated to determine their function. A carbohydrate ligand that modifies the function of a leczyne-expressing cell is one that when contacted with the cell results in a function  
25 that differs from the function of the same type of cell that had not contacted the ligand.

As used herein, "function" in reference to a cell includes any activity that can be detected for a cell.

The function of a cell can vary with the nature of the cell in question. For example, the function of a T lymphocyte can include activities such as the production of certain cytokines, acquisition of cell mediated lympholysis, ability to mediate antibody dependent cell mediated cytotoxicity or the ability to help B lymphocytes produce antibody. Thus, a particular carbohydrate ligand that can bind to a leczyne on a T lymphocyte and subsequently effect the function of the cell can do so by increasing or decreasing any of the above T lymphocyte functions. As another example, addition of a complex carbohydrate to the epithelial cells of the gastrointestinal tract will affect iron absorption.

Contacting a carbohydrate ligand with a leczyne-expressing cell can be performed *in vitro* in a cell culture medium. Methods for measuring the function of lymphoid cells or other cells are well known in the art (see for example, Colligan et al., Curr. Protocols in Immunol. (Greene Publishing Associates and Wiley Interscience, New York, 1992); Mishell and Shiigi, Selected Meth. in Cell. Immunol. (W. H. Freeman and Co., New York, 1980), each of which are incorporated herein by reference).

Methods described above for identifying a carbohydrate ligand that modifies the function of a leczyne-expressing cell are also useful for identifying a leczyne that modifies the function of a carbohydrate ligand-expressing cell.

This page

Blank in  
originals  
ordered.

compared to the amount of carbohydrate ligand that bound to leczyne in a control sample that did not contain peptide. If the amount of carbohydrate ligand that bound to the leczyne in the sample containing peptide is less than the amount of carbohydrate ligand that bound to the leczyne in the control sample, then it can be concluded that the peptide had bound to the carbohydrate ligand binding site of the leczyne and is therefore a peptide mimetope of the carbohydrate ligand.

10        A peptide mimetope can be identified in an assay format that utilizes a carbohydrate ligand containing a detectable label and a leczyne that is bound to a solid support or is expressed by a cell. Methods disclosed herein for identifying a carbohydrate ligand that bind to  
15 a leczyne are useful to generate the assay format for identifying a peptide mimetope of a carbohydrate ligand.

         A defined peptide sequence can be chemically synthesized or produced by biological methods, such as by recombinant DNA techniques (see, for example, Sambrook et al., *supra*, 1989). A complex mixture of peptides also  
20 can be used to identify a peptide mimetope. Such complex mixtures can include, for example, a mixture of defined sequences, or can be a semi-random or random library of sequences. Methods to generate peptide libraries by such  
25 methods as chemical synthesis on a bead or a microtiter plate or biological production such as on the surface of a bacteriophage are well known in the art (see, for example, Huse et al., Science 246:1275-1281 (1989), which is incorporated herein by reference).

A peptide that can bind to the carbohydrate ligand binding site of a leczyne can also have some of the functional characteristics of a carbohydrate ligand and thus be considered a functional mimetope of the  
5 carbohydrate ligand. Such peptide mimetopes can be used to modify the function of a cell and also can be used to treat a disease state that involves a leczyne that can bind to the mimetope.

A cell can be modified to produce a carbohydrate  
10 ligand by introducing an expression vector encoding a leczyne into a cell to obtain expression of the leczyne, which results in production of the carbohydrate ligand by the cell. Cells producing a particular carbohydrate ligand are useful to provide unique types of ligands,  
15 which can be purified from the cells. In addition, such cells are useful in binding assays to identify a leczyne that binds the ligand.

An immune response in an individual, such as a human or other animal, can be modulated using an antigen for  
20 which the immune response is desired and a carbohydrate ligand that binds to a leczyne. As leczyne include, for example, the major histocompatibility complex molecules that are involved in the immune system, injection of a carbohydrate ligand and an antigen can modulate an immune  
25 response. As used herein, "modulate" means increase or decrease. An increase in the immune response can be obtained by administering a carbohydrate ligand bound to

antigen such that the antigen is targeted via the leczyne to an antigen presenting cell.

An antigen can be associated with a carbohydrate ligand by covalently bonding the antigen to carbohydrate  
5 or to any protein or lipid of the ligand using methods well known in the art. The actual method to covalently couple the antigen to the carbohydrate ligand will depend on the nature of each molecule to be coupled and whether the coupling procedure is detrimental to the any critical  
10 antigenic determinants of the antigen or the capability of the carbohydrate ligand to bind its' target leczyne. Such detrimental effects can be readily evaluated in binding assays as described above.

More than a single antigen molecule or more than a  
15 single carbohydrate ligand can be coupled together to produce an immunogen. Such molecules can be made multivalent for either or both of the antigen or the carbohydrate ligand and can be used for eliciting a greater immune response than an immunogen containing a  
20 single molecule of antigen and a single molecule of a carbohydrate ligand.

Methods to increase an immune response in an individual are well known to those in the art and require optimization of parameters such as dose, route of  
25 administration, use of an adjuvant, or schedule of administration (see, for example, Harlow and Lane, chapter 5, *supra*, 1988). These same methods can be adapted for use with carbohydrate ligands. An increased

immune response obtained after administering an antigen and a carbohydrate ligand is achieved when the immune response parameter has increased by a statistically significant level over the level of the parameter  
5 manifested prior to administration of the antigen and carbohydrate ligand.

The immune response parameters that can increase after administering an antigen associated with a carbohydrate ligand include an antibody-mediated response  
10 or a cellular-mediated response. Methods to measure antibody immune responses are well known to those in the art and include, for example, detection of immunoglobulins by both *in vitro* and *in vivo* methods (see for example Harlow and Lane, *supra*, 1988). Methods to  
15 measure cellular immune responses are also well known in the art and include *in vivo* methods such as skin testing for delayed hypersensitivity and *in vitro* methods such as direct cell cytotoxicity or cell activation assays (see, for example, Colligan et al. *supra*, 1992; Mishell and  
20 Shiigi, *supra*, 1980).

An antigen associated with a carbohydrate ligand can also be used to decrease an immune response to the antigen and can be particularly useful for treating a deleterious immune response such as an autoimmune disease  
25 state. Methods for decreasing an immune response can, under some conditions result in a prolonged state of specific immunological unresponsiveness to the antigen, commonly referred to as a state of tolerance to the antigen.

Decreasing an immune response to an antigen by administering the antigen bonded to a carbohydrate ligand can be accomplished using methods well known in the art to suppress or tolerize an individual to an antigen.

- 5 Such methods include, for example, administration of low doses, monomeric and nonaggregated forms of the antigen and carbohydrate ligand or administration orally. In addition, a decreased immune response can be obtained by administering the antigen and carbohydrate ligand
- 10 concurrently with an immunosuppressive agent such as cyclosporin A, FK506 or antibodies to a particular T lymphocyte cell-surface receptor. Methods for using such agents to decrease the immune response to an antigen in humans or animals are well known in the art.
- 15 A disease state involving a leczyne can be treated by administering an effective amount of a carbohydrate ligand that binds to the leczyne. As used herein, the term "disease state" includes any diseases, whether genetic or acquired, provided a leczyne plays a role in
- 20 the disease process. Disease states which may be ameliorated using methods described herein include inflammation, transplantation rejection, and may also includes diseases having both a genetic and an environmental basis such as iron storage diseases,
- 25 autoimmunity or cancer. In addition, other disease states which may be ameliorated using methods described herein include those resulting from an infectious agent such as a virus, bacteria, yeast or parasite. The ability of an infectious agent to enter and infect cells
- 30 of the host can occur by binding to the leczyne or



carbohydrate ligand expressed on the cells of the host. A peptide mimetope for a carbohydrate can also be used to treat a disease state that involves a leczyne for which the mimetope can bind.

5        A disease state involving a leczyne can be treated by administering a leczyne having a similar binding specificity for a carbohydrate ligand as the leczyne involved in the disease state. The disease states useful for treatment by a leczyne include those described above  
10 for treatment by a carbohydrate ligand. Thus, aberrant self-recognition, mediated by a leczyne in a diseased individual, can be treated by administration of a leczyne. Such a leczyne can bind to the natural carbohydrate ligand detected on a target cell by the  
15 aberrant self-reactive leczyne-expressing cell, and, therefore, block the ability of the self-reactive leczyne-expressing cell to recognize and react aberrantly towards the target cell.

      An iron metabolic disorder known as hemochromatosis  
20 can also be treated using methods disclosed herein because defects in iron metabolism can have a basis in leczyne function. In elevated concentrations, iron is a toxic inorganic molecule that has been implicated in the pathophysiology of a number of common diseases. These  
25 include but are not limited to cancer (Stevens et al, N. Engl. J. Med., 319:1047 (1988); Stevens, et al., Med. Oncol. Tumor Pharmacother, 7:177-181 (1990)), heart disease (Kannel, et al, 1976; Sullivan, Lancet, 1:1293-1294 (1981); Salonen, et al, Circulation,

86:803-811 (1992)), reperfusion injury (Zweier, J. Biol. Chem., 263:1353-1357 (1988)) and rheumatoid arthritis (Blake et al., Arthritis Rheum., 27:495-501 (1984)).

There is no argument that severe iron overload results in  
5 a constellation of pathologies, collectively called hemochromatosis, the most common genetic disease known to affect humans.

Hemochromatosis results from enhanced absorption of iron from the GI tract by active transport but the gene  
10 or genes causing the underlying metabolic defect is currently unknown. Identification of the genes responsible for the absorption of iron, and developing an animal model in which iron overload is due to active enhanced absorption of iron from the GI tract, would  
15 greatly facilitate understanding hemochromatosis and increase knowledge about the general mechanisms of iron metabolism. As disclosed herein, an MHC-encoded lecyzme may be involved in the pathogenesis of hemochromatosis.

Hemochromatosis is not usually brought to clinical  
20 attention until symptoms develop, and several studies have indicated that removal of the iron after the development of tissue damage does not necessarily improve the organ function (Cundy, et al., Clin. Endocrinol., 38:617-620 (1993); Westera et al., Am. J. Clin. Path.,  
25 99:39-44 (1993)). Hemochromatosis is an underdiagnosed and undertreated disease that would benefit greatly from early diagnosis and an effective treatment (for reviews see Edwards et al., Hosp. Pract. Suppl., 3:30-36 (1991);

Edwards and Kushner, N. Engl. J. Med., 328:1616-1620 (1993)).

Untreated hemochromatosis is characterized by iron overload of parenchymal cells, which is toxic and in many cases a probable cause of various complications including hepatopathy (including cirrhosis, and liver cancer), arthropathy, hypogonadotropic hypogonadism, marrow aplasia, skin disorders, diabetes mellitus, and cardiomyopathy (for review see Halliday and Powell, Iron and Human Disease, Lauffer, RB, (ed). 131-160 (1992)).

There are reportedly 1.5 to 2 million active cases of hemochromatosis within the U.S., with approximately 25% of late diagnosed or untreated patients developing hepatomas.

In untreated hemochromatosis, iron is universally deposited in the hepatocytes of the liver, and elevated saturation of transferrin with elevated serum ferritin levels combined with liver biopsy provides the best diagnostic test currently available (Fairbanks, Hosp. Pract., 26:17-24 (1991)). The iron is found primarily in the cytoplasm of hepatocytes, and by electron microscopy in lysosomal vacuoles, and in more severe cases, iron is deposited in mitochondria (for review see Iancu, Ped. Pathol., 10:281-296 (1990)). Other liver toxins such as alcohol and hepatitis exacerbate the damage caused by the iron deposition (Piperno et al., J. Hepat., 16:364-368 (1992)). Patients with hemochromatosis are advised not to drink alcohol, because of increased liver damage, or

to smoke tobacco products, as iron deposition can also occur in the lungs.

Hemochromatosis is reported in the literature as an autosomal recessive disease in which the responsible  
5 gene(s) is linked to the A locus of the human MHC (HLA complex), located on human chromosome 6 (Simon and Brissot, Hepatol., 6:116-124 (1988)). Linkage to human HLA-A3 has been documented in approximately 73% of cases. However, other genetic loci also have been implicated,  
10 especially in African (Gorduke et al., N. Engl. J. Med., 326:95-100 (1992)) and African-American populations (Barton et al., Blood, 85:95a (1993)).

Hemochromatosis is presently recognized as the most common genetic malady in humans, far exceeding cystic  
15 fibrosis, phenylketonuria and muscular dystrophy combined (Leggett et al., Clin. Chem., 36:1350-1355 (1990)). One explanation for the high incidence of this genetic disease may be that mutations in multiple linked genes produce a similar phenotype. Hemochromatosis occurs most  
20 frequently in populations of European origin with a frequency in homozygotes and heterozygotes of approximately 0.3 and 13%, respectively.

Several markers, including the recently described D6S105, have been identified in the human MHC locus and  
25 have narrowed the genomic location of the hemochromatosis gene to within 1 centimorgan of the A locus (Jazwinska et al., Am. J. Hum. Genet., 53:347-352 (1993)), and possibly centromeric to HLA-F (Gasparini, et al., Hum. Mol.

Genet., 5:571-576 (1993)). Others have reported candidate hemochromatosis genes located 20-200 kb telomeric to HLA-A (el Kahloun et al., Hum. Mol. Genet., 2:55-60 (1993)). While several of these candidate genes  
5 were thought to be single copy, three of the genes, termed HCG II, IV and VII, were found to be multicopy genes. Thus, despite the advances made in determining the location of the hemochromatosis gene, it has not yet been isolated.

10 Most animal models for iron overload are not entirely suitable for the study of hemochromatosis since they do not reflect enhanced iron absorption from the gut by active transport. Mice homozygous for deletion of the gene encoding  $\beta_2M$  ( $\beta_2$ -/-mice (Koller et al., Science,  
15 248:1227-1230 (1990); Zijlstra et al., Nature, 344:742-746 (1990)), however, provide an excellent animal model for the study of hemochromatosis. These animals lack detectable class I proteins on the cell-surface, although biochemical labeling shows that class I gene  
20 products are being synthesized. Activated lymphocytes from  $\beta_2$ -/- animals can be lysed by activated natural killer (NK) cells, again suggesting a deficiency in class I expression (Liao et al., Science, 253:199-202 (1991)). These mice were originally developed to study the role of  
25  $\beta_2M$  in development. While the mice developed and bred normally, they failed to generate significant numbers of CD8+ T cells. Consequently, these mice have been intensely studied from an immunologic perspective.

$\beta_2$ -/-mice combat viral infections relatively well, although the course of the infection is longer than in normal animals (Eichelberger et al., J. Exp. Med., 174:875-878 (1991); Muller et al., Nature, 255:1576-1579 (1992)). They reject allografts (Zijlstra et al., J. Exp. Med., 175:885-889 (1992)) and show higher levels of Ig production and faster class switching of antibody types than normal mice. Although CD8+ T cells are low to undetectable at birth, studies have shown that the animals can generate CD8+ T cells, and a cytotoxic CD8+ T cell response can be mounted under appropriate circumstances (Apasov and Stikovsky, J. Immunol., 152:2087-2097 (1994)). Another significant abnormality reported in these animals is that they develop hyperglycemia (glucose>300 mg/dl) in old age (greater than 2 years). It has been suggested that the onset of diabetes in the  $\beta_2$ -/- mice is related to autoimmunity (Faustman et al., Science, 254:1756-1761 (1991)), however this explanation has been disputed (Serreze et al., Diabetes, 43:505-509 (1994); Wicker et al., Diabetes, 43:500-504 (1994)).

$\beta_2$ -/-mice can develop iron overload that is similar to human hemochromatosis.  $\beta_2$ -/-mice can spontaneously develop hepatomas. This observation combined with the molecular biology data of the  $\beta$ -GAP genes (see Example I), suggested that the mice would develop iron overload. Histochemical examination of tissues from these mice, confirmed this hypothesis. Iron was found deposited in the liver of all animals, and in the kidneys, spleen and lungs of some of the animals. In addition, 16% of the

animals developed liver disease, having either hepatomas or liver necrosis. Thus, the clinical findings for the  $\beta_2$ -/- deficient mice are sufficiently similar to the pathology of hemochromatosis to make the  $\beta_2$ -/-mouse an

---

organ-specific autoimmune diseases such as Addison's disease, Goodpasture's syndrome, Grave's disease, Hashimoto's thyroiditis, idiopathic thrombocytopenia purpura, myasthenia gravis or pernicious anemia. As  
5 hemochromatosis in humans is likely mediated by a  $\beta$ -GAP promoter-driven leczyne, then treatment with a carbohydrate ligand, leczyne or competing molecule with the same or similar binding specificity as the leczyne involved in the disease may be used to modulate the  
10 disease process. A carbohydrate ligand that binds the nonclassical class I leczyne involved in hemochromatosis can be administered to inhibit binding to the  $\beta$ -GAP iron carrier.

A process to follow for using a carbohydrate ligand  
15 to treat a disease such as autoimmunity can first require identification of the leczyne that is involved in the disease process. Subsequently, a candidate carbohydrate ligand that can bind to the leczyne is identified by methods disclosed herein. Thus, such candidate  
20 carbohydrate ligands can then be tested *in vitro* to identify those efficient at blocking the autoimmune reaction exhibited when the leczyne on autoreactive immune cells from the diseased individual recognizes a carbohydrate molecule expressed on the cells of the  
25 individual that is the target of the autoreactive cell. The autoimmune reaction can be measured by an increase in a cell function such as cell proliferation or release of cytokines (see for example, Colligan et al. *supra*, 1992; Mishell and Shiigi, *supra*, 1980). The best candidate



carbohydrate ligands can then be used as a medicament to treat the disease.

The methods disclosed herein for the treatment of hemochromatosis are also suitable for the treatment of many other medical diseases or complication resulting from iron overload. Since multiple leczyme genes are involved in mediating control of iron metabolism, the type of mutation, its location in the gene and the number and type of leczyme genes mutated in an individual are factors that can effect the extent of iron overload in an individual. As the extent of iron overload exhibited by an individual is dependent on the above factors, then the methods disclosed herein to treat hemochromatosis are also applicable for treating other diseases resulting from iron overload. Such diseases include, for example, hepatopathy (including cirrhosis, and liver cancer), arthropathy, hypogonadotropic hypogonadism, marrow aplasia, skin disorders, diabetes mellitus, and cardiomyopathy (for review see Halliday and Powell, Iron and Human Disease, Lauffer, RB, (ed). 131-160 (1992)).

In order to modulate hemochromatosis or other iron storage disease, the carbohydrate ligand or mimetope is administered in an effective amount. The total effective amount can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which the multiple doses are administered over a more prolonged period of time. One skilled in the art would know that

the concentration of a carbohydrate ligand required to obtain an effective dose in a subject depends on many factors including the age and general health of the subject as well as the route of administration and the  
5 number of treatments to be administered and the chemical form of the carbohydrate ligand. In view of these factors, the skilled artisan would adjust the particular amount so as to obtain an effective amount for the subject being treated.

10 A carbohydrate ligand or a leczyne can also be useful in vivo for the treatment of autoimmune diseases involving a leczyne. In autoimmune disease, a leczyne expressed on a lymphoid cell can recognize a self-carbohydrate ligand as a foreign carbohydrate  
15 ligand, resulting in immune-directed destruction of cells expressing the self-carbohydrate ligand. Thus, administration of a carbohydrate ligand, mimetope or other competing molecule that can bind to the leczyne involved in aberrant self-recognition can block lymphoid  
20 cell recognition or activation leading to a reduction in symptoms or cessation of autoimmune disease. Alternatively, administration of a leczyne that has the same or similar binding specificity for the self-carbohydrate ligand recognized by a leczyne of the  
25 autoreactive lymphoid cell can also be used to treat the autoimmune disease.

A carbohydrate ligand or a leczyne can be used to treat a disease state resulting from an infectious agent such as a virus, bacterium, yeast or parasite.

Infectious agents have evolved to express their own external receptors that can recognize carbohydrate structures or leczyms on the cell-surface, enabling entry of the agent into the cell to be infected. Thus, administration of an appropriate carbohydrate ligand or a leczyms to an individual exposed to an infectious agent can block the binding of the agent to target cells, subsequently inhibiting the extent of infection and thereby reducing the spread of the disease.

10

A carbohydrate ligand or a leczyms of the present invention can be used to treat transplantation rejection. Since rejection is based on the recognition of foreign molecules by lymphocytes of the transplant recipient, then treatment with a carbohydrate ligand that can bind to the leczyms of the transplant recipient's lymphocyte that is involved in foreign antigen recognition can inhibit recognition leading to transplantation rejection. Also, administration of a leczyms that has the same or similar binding site specificity as the leczyms of a transplant recipient's lymphocyte involved in foreign antigen recognition can inhibit recognition leading to transplantation rejection.

A carbohydrate ligand or leczyms of the present invention is particularly useful when administered as a pharmaceutical composition containing a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include, for example, aqueous solutions such as a physiologically buffered saline or other solvents or vehicles such as glycols,

30

glycerol, oils such as olive oil or injectable organic esters.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize or to increase the absorption of a carbohydrate ligand or leczyne. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose, dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the composition.

One skilled in the art would know that a pharmaceutical composition containing a carbohydrate ligand or leczyne can be administered to a subject by various routes including, for example, by direct instillation, orally or parenterally, such as intravenously, intramuscularly, subcutaneously or intraperitoneally. The composition can be administered by injection or by intubation. The pharmaceutical composition also can be incorporated, if desired, into liposomes or microspheres or can be microencapsulated in other polymer matrices (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, Boca Raton, FL, 1984), which is incorporated herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and

metabolizable carriers that are relatively easy to make and administer.

An expression vector encoding a leczyne can be administered *in vivo* to treat a disease state resulting from a leczyne. For example, a disease state resulting from a mutated leczyne, such as anemia, can be treated by administering an expression vector encoding a functional leczyne involved in iron transport and obtaining expression of the vector in cells of the digestive tract.

10 The level of expression of a particular leczyne in a cell can have a impact on the nature of a carbohydrate ligand expressed by the cell. If expression of a particular carbohydrate ligand is involved in a disease process, the ligand can be eliminated from a cell by  
15 reducing the expression of the leczyne responsible for producing the ligand. Thus, an expression vector can contain an exogenous nucleic acid molecule encoding an antisense nucleotide sequence that is complementary to a nucleotide sequence encoding a portion of a leczyne such  
20 that when introduced into a cell under the appropriate conditions, the expression vector can produce an antisense nucleic acid molecule, which can selectively hybridize to the leczyne gene or message in a cell and, thereby, affect the expression of the leczyne in the  
25 cell. For example, the antisense nucleic acid molecule can hybridize to a leczyne gene in the cell and can reduce or inhibit transcription of the leczyne gene. Also, the antisense molecule can hybridize to the message encoding the leczyne in the cell and can reduce or

inhibit translation, processing and cell stability or half-life of the RNA.

Expression vectors also can be used to effect the expression of a leczyne or of a carbohydrate ligand involved in a disease state by introducing into a cell an exogenous nucleic acid molecule encoding a ribozyme that can specifically cleave RNA encoding the leczyne or peptide backbone of a carbohydrate ligand. Thus, by introducing the ribozyme into cells involved in a disease process, one can reduce expression of the leczyne or carbohydrate ligand involved in the disease and therefore reduce or inhibit the disease process. An antisense nucleic acid molecule or a ribozyme can be chemically synthesized and incorporated into an expression vector using recombinant DNA techniques. The antisense nucleic acid molecule or ribozyme also can be added directly to a cell without having been incorporated into an expression vector.

Methods for introducing an expression vector into cell are well known in the art. Such methods are described in Sambrook et al, *supra*, 1989; Kriegler M. Gene Transfer and Expression: A Laboratory Manual (W. H. Freeman and Co. New York NY (1990), both of which are incorporated herein by reference) and, for example, include transfection methods such as calcium phosphate, electroporation, lipofection, or viral infection.

Recombinant viral vectors are available for introducing an exogenous nucleic acid molecule into a

mammalian cell and include, for example, adenovirus, herpesvirus and retrovirus-derived vectors. For example, a viral vector encoding a leczyne can be packaged into a virus to enable delivery of the genetic information and  
5 expression of these leczyne in gastrointestinal epithelial cells following infection by the virus. Also, a recombinant virus which contains an antisense sequence or a ribozyme specific for a nucleotide sequence encoding a leczyne can introduced into a cell in an individual to  
10 inhibit a disease state mediated by the leczyne or a leczyne with a similar carbohydrate binding specificity.

Recombinant viral infection can be more selective than direct DNA delivery due to the natural ability of a virus to infect only certain types of cells. This  
15 natural ability for selective viral infection can be exploited to limit infection to only certain cell types within a mixed cell population. For example, adenoviruses can be used to restrict viral infection principally to cells of epithelial origin. In addition,  
20 a retrovirus can be modified by recombinant DNA techniques to enable expression of a unique receptor or ligand that provides further specificity to viral gene delivery. Retroviral delivery systems that provide high infection rates, stable genetic integration and high  
25 levels of exogenous gene expression are well known in the art.

As described above, recombinant viral delivery systems exist that provide the means to deliver genetic information into a selected type of cell. The choice of

viral system will depend on the desired cell type to be targeted, while the choice of vector will depend on the intended application. Recombinant viral vectors are readily available to those in the art and can be easily  
5 modified by one skilled in the art using standard recombinant DNA methods (see, for example, Krieger, Gene Transfer and Expression: A Laboratory Manual, (W.H. Freeman and Company, 1990); Goeddel, Methods in Enzymology, vol. 185, (Academic Press, 1990); and Stoker,  
10 In Molec. Virol., A Practical Approach (eds. Davison and Elliott, IRL Press, 1993), all three of which are incorporated herein by reference).

A genetic predisposition for hemochromatosis or other iron storage diseases based on a leucocyte can be  
15 diagnosed by detecting a mutation in the heavy chain of a class I MHC molecule encoded for by a gene in the MHC locus. These methods can be used to diagnose an individual having the symptoms of an iron storage disease. A positive diagnosis of mutation in an  
20 individual's heavy chain is useful to verify the underlying cause of the disease and by identifying the particular leucocyte that is mutated. The identification of the mutated leucocyte can be used with the methods disclosed herein to identify a carbohydrate suitable for  
25 treating the disease.

An individual who does not have an iron storage disease, but is suspected of inheriting a mutation that can predispose the individual to develop an iron storage



disease later in life can also benefit from testing for mutations by the methods disclosed herein.

A mutation that is diagnostic for the disease is one that results in a significantly reduced affinity of the heavy chain for human  $\beta_2\text{M}$ . For example, a mutation in a nonclassical class I heavy chain that results in deletion of a signal for phosphorylation is a mutation that is diagnostic for hemochromatosis since a properly phosphorylated heavy chain is necessary for the chain to interact with  $\beta_2\text{M}$ . Consensus amino acid sequences that signal a cell to phosphorylate a serine or a threonine residue in a polypeptide are well known in the art. A mutation that is diagnostic for hemochromatosis also can occur in a region of the heavy chain that is near to a phosphorylation site. Such a mutation can reduce the ability of the heavy chain to associate with  $\beta_2\text{M}$  if the phosphate group added to this site cannot be removed in a cell.

Methods to detect a phosphorylation site mutation in a nonclassical class I heavy chain can be based either on analysis of the protein or the nucleic acid encoding the protein. For protein determination, the nonclassical class I molecule can be purified from a source of cells or body fluids of an individual and the heavy chain can be isolated from  $\beta_2\text{M}$ . Methods to purify a class I MHC molecule and isolate the heavy chain from  $\beta_2\text{M}$  are well known in the art. The isolated heavy chain can then be subjected to amino acid sequencing, peptide mapping or other such protein analyses to determine if the sequence

a phosphorylation site has been mutated. Such methods for protein determination are well known to those in the art.

A mutation in a nucleic acid sequence can be  
5 detected by various methods to analyze nucleic acids such as by nucleic acid sequencing, polymerase chain reaction or hybridization. Such methods are well known to those in the art (see, for example, Sambrook et al, *supra*, 1989; Hames and Higgins Nucleic Acid Hybridisation: a  
10 practical approach (IRL Press, New York, 1985), both of which are incorporated herein by reference).

Methods to detect decreased binding of a mutated heavy chain with  $\beta_2M$  can be used for diagnosing an iron storage disease such as hemochromatosis. In these  
15 methods, the heavy chain of an class I MHC molecule is isolated from an individual and contacted with  $\beta_2M$  under conditions suitable for a non-mutated such heavy chain to associate with  $\beta_2M$ . A control reaction, which contains a non-mutant form of the same or similar class I heavy  
20 chain to the one being tested for a mutation is performed in parallel. After contacting the heavy chain with  $\beta_2M$ , the reaction is incubated under suitable conditions, including, for example, an appropriate buffer concentration and pH and time and temperature, which is  
25 sufficient for the control heavy chain to associate with  $\beta_2M$ . The heavy chain being tested from the individual is considered to have a mutation diagnostic for an iron storage disease when the fraction of this heavy chain

that associates with  $\beta_2M$  is significantly less than the fraction of control heavy chain that associates with  $\beta_2M$ .

The association of a class I heavy chain with  $\beta_2M$  can be detected, for example, by attaching one of the  
5 molecules to a solid support and attaching a detectable label such as a radionuclide or a fluorescent label to the other molecule and measuring the amount of detectable label that is associated with the solid support, wherein the amount of label detected indicates the amount of  
10 association of the heavy chain with  $\beta_2M$ .

The following examples are intended to illustrate but not limit the present invention.

### EXAMPLE I

#### CLONING AND EXPRESSION OF THE $\beta$ -GAP GENES

15 This example provides an approach to identify and clone leucocyte genes from various species of animal to elucidate their role in iron metabolic diseases.

#### Cloning of the mouse $\beta$ -Gap Genes

Genomic  $\alpha$  libraries were constructed by partial Hae  
20 III digestion of DNA from A/J and Balb/c mouse liver and cloning the fragments into the vector Charon 4A. The libraries were screened with the S15 probe, isolated from the H-2L<sup>d</sup> gene (Margulies et al., Nature, 295:168-170 (1982), which is incorporated herein by reference)). S15

is a 3' class I MHC mouse probe and consists of 522 base pairs including 36 base pairs of exon 4 encoding the alpha-3 domain and 486 base pairs of intron (Evans et al., Proc. Natl. Acad. Sci. (USA), 79:1994-1998 (1982)).

5 Probes were prepared by excising the insert from M13 RF or pUC18, purifying the fragment from disulfide cross-linked acrylamide gels (Hansen, Anal. Biochem. 116:146-151 (1981)), and labeling with <sup>32</sup>P to a specific activity of >10<sup>8</sup> cpm/μg by nick translation (Rigby et  
10 al., J. Mol. Biol. 113:237-251 (1977)). Libraries were screened using standard colony hybridization techniques (for details see Sambrook et al., *supra*, 1988).

Seventeen unique α clones were isolated from the libraries and were subjected to restriction enzyme  
15 digestion mapping. BamHI digestion and gel electrophoresis of these clones revealed five from the A/J strain and one from the Balb/c strain that contained a unique 500 base pair (bp) BamHI restriction fragment (BB500). The six clones containing the unique fragment  
20 were subjected to BAMHI digestion, the BB500 fragment was gel purified and subcloned into M13 vector Mp18 and mp19 (Yanisch-Perron et al., Gene 33:103-119 (1985)). DNA sequences were determined by the chain termination method (Sanger, et al., Proc. Natl. Acad. Sci. (USA)  
25 74:5463-5467 (1977)) using <sup>35</sup>S-ATP. Reactions were analyzed on 6% urea-polyacrylamide gradient gels (Biggins et al., Proc. Natl. Acad. Sci. (USA) 80:3963-3965 (1983)). DNA sequences were assembled and analyzed using the University of Wisconsin Computer Group Programs

(Devereux, et al., Nucleic Acids Res. 12:387-395 (1984))  
run on a VAX-11/785 computer.

DNA sequence comparisons demonstrated that the BB500 fragments share greater than 93% sequence homology. A  
5 region within the BB500 fragment shows 100% sequence homology between the  $\alpha$  clones and has been termed  $\beta$ -GAP (globin analogous promoter) since it is a regulatory motif that shares sequence homology with mouse, rabbit or human  $\beta$ -globin promoters (for a detailed comparison see  
10 ahead). There is close similarity between all six fragments (called  $\beta$ -GAP1-6) with the minor exception of  $\beta$ -GAP4 where an 8 base pair sequence AAGAGGAG, immediately downstream of a CCAAT element, has been deleted. There are other minor differences between these  
15 sequences, and the  $\alpha$  clones they have been isolated from demonstrate different restriction patterns confirming that the various  $\alpha$  clones contain unique sequences and are not a cloning artifact. Thus, the A/J strain mouse contains at least five highly homologous  $\beta$ -GAP sequences  
20 within its genome.

#### Mapping the $\beta$ -GAP Sequences Map to Chromosome 17 in the Mouse

Southern blotting was used to determine if the BB500 sequence could identify genes located on chromosome 17 of  
25 the mouse. DNA from several Chinese hamster ovary (CHO) mouse somatic hybrid cell lines were evaluated by Southern blotting using the  $\beta$ -GAP6 BB500 probe. Genomic DNA was isolated from cultured cells, digested with

EcoR1, electrophoresed on 0.8% agarose gels and transferred to a nitrocellulose membrane. Hybridization with the BB500 probe was carried out in the presence of dextran sulfate under the conditions described by Meinkof and Wahl (Anal. Biochem. 138:267-284 (1985)) with a final wash in 0.2X sodium chloride sodium citrate buffer, pH 7.0 (SSC) at 60°C.

The BB500 probe hybridized with the HM27 cell line containing the DNA from mouse chromosomes 15 and 17 and revealed the same banding pattern as with total genomic BALB/c DNA. The cell line HM65 that lacks BALB/c chromosome 17 was devoid of hybridizable bands, indicating that the probe did not bind nonspecifically to CHO DNA. DNA from other CHO cell lines containing mouse chromosomes other than chromosome 17 were examined by Southern blotting with the BB500 probe and were found to be negative (not shown). These results indicate that the  $\beta$ -GAP sequences all map to chromosome 17 in the mouse.

#### Mapping the $\beta$ -GAP Sequences to the Murine O/TL Complex

The fact that the  $\beta$ -GAP sequences were isolated from the mouse genome provided several powerful tools to precisely map the location of the sequences. First, the murine MHC is highly characterized, particularly with respect to the nonclassical class I region and, secondly, congenic strains of mice exist where the position of genes in the MHC can be pinpointed. Congenic strains were originally developed by breeding strains of inbred mice together. Subsequent generations of chromosomal

crossing over has produced a number of strains which contain a portion of the MHC from one strain and the remainder of the MHC from another strain. Consequently, it is possible to compare restriction fragment length polymorphism (RFLP) between the strains, and determine if the banding patterns are linked to a given MHC locus (for review see Klein, Natural History of the Major Histocompatibility Complex, 50-73 (1986)). RFLP analysis was performed by obtaining purified genomic DNA from the various mouse strains, digesting the DNA with EcoRI and performing Southern hybridization with the  $\beta$ -GAP6 BB500 probe as described above. The Southern blot showed that the probe identified up to ten different bands from the DNA of the mouse strains tested (Table 1). Four of these bands, 30 kb, 20 kb, 16 kb and 10.5 kb, were mapped within the MHC locus. The RFLP analysis indicated that there were at least four to six copies of the  $\beta$ -GAP sequences/genome depending on the strain of mouse tested. In addition genetic analysis of the RFLP patterns indicated that the 30 kb and 10.5 kb  $\beta$ -GAP bands mapped to Q region between Q1 and Q4 while the 20 kb and 16 kb  $\beta$ -GAP bands mapped to the T region. In addition, two of the  $\beta$ -GAP sequences that did not demonstrate RFLP polymorphism were mapped telomeric to the classical class I genes.

#### Locating the $\beta$ -GAP Sequences Directly Adjacent to Nonclassical Class I Genes

The two of the  $\beta$ -GAP gene sequences that were mapped to the Q region between Q1 and Q4, were directly linked

to Q1 and Q2 by DNA sequence analysis of Q1 and Q2 genes isolated from a C57BL/6 (H-2<sup>b</sup>)  $\alpha$  library. Sequencing showed that both the Q1 and Q2 genes are associated in a head to head configuration with an unknown gene

5 (currently defined as the  $\beta$ -GAP gene) with both genes transcriptionally regulated by a single promoter/enhancer region having two promoters defined by a pair of CAAT and TATA boxes located about 25 bp apart on opposite strands of the DNA. Thus, having intact promoters and a common  
10 regulatory region, the class I and  $\beta$ -GAP genes would be transcribed from opposite strands, with the class I genes Q1 or Q2 transcribed from 5' to 3' on the top strand and the  $\beta$ -GAP gene transcribed from 5' to 3' on the bottom strand.

15 The sequence analysis of Q1 and Q2 genes from C57BL/6, as well as a TL gene from A/J (H-2<sup>a</sup>, Watts et al. EMBO J. 8:1749-1759 (1980)) indicated that  $\beta$ -GAP promoter and regulatory regions had replaced the typical classical class I-type 5' regulatory sequences known to be involved  
20 in the regulation of classical class I genes. The  $\beta$ -GAP promoter is an active promoter since it is known that the Q2 gene expresses a gene product that can be detected in the intestine (Wang et al., Immunogenet., 38:370-372 (1993)). These results indicate that the  $\beta$ -GAP promoter  
25 regulates the expression of some nonclassical class I genes.



Table 1

COMPARISON OF SOUTHERN BLOT ANALYSIS OF EORI DIGESTS OF MURINE DNA USING THE BB500 LOW COPY NUMBER PROBE WITH  
GENETIC MAPS OF VARIOUS ALLOGENEIC AND CONGENIC STRAINS.

STRAIN	MHC REGION  K D Q T	EcoRI BAND SIZE (kb)										
		30	20	16	14.5	13	10.5	9.2	8.0	7.8	7.5	
B6,B10	b b b b	+		+						+	+	
B6,K1	b b k k			+			+			+	+	
B6,K2	b b bk k	+		+						+	+	
AKR	k k k k			+			+			+	+	
B6,K3	k k b a	+	+		+					+		
B6,K4	k k k a		+		+		+			+	+	
B6-H-2 <sub>k</sub>	k k k k			+			+			+	+	
B6-T1a <sub>o</sub>	b b ba a	+	+		+					+	+	
A/J	k d a a		+		+				+	+	+	
Balb/cJ	d d d d		+		+		+			+	+	
B10,A	k d a a		+		+		+		+	+	+	
A-T1a <sub>b</sub>	k d a b			+			+			+	+	

Head to head gene structure with co-regulation of the genes has been previously described in organisms ranging from bacteria to humans, indicating that co-regulation is a widely adopted strategy. (Brickman et al., J. Molec. Biol., 212:669-682 (1990); Xu and Doolittle, Proc. Natl. Acad. Sci. (USA), 87:2097-2101 (1990); Lennard and Fried, Molec. Cell. Biol., 11:1281-1294 (1991); Heikkila et al., J. Biol. Chem., 268:24677-24682 (1993); Fererjian and Kafatos, Dev. Biol., 161:37-47 (1994); Sun and Kitchingman, Nucleic Acids Res., 22:861-868 (1994)). In both prokaryotic and eukaryotic systems, interaction between, or linkage in a metabolic pathway of two gene products has been suggested (Galvalas, et al., Mol. Cell. Biol., 13:4784-4792 (1993); Lightfoot et al., Br. J Cancer, 69:264-2673 (1994)). It should be noted that in the  $\beta$ -GAP clones so far studied, the Q1 and Q2 genes still possesses their own CAAT and TATA elements, and it is only the typical classical class I regulatory enhancer regions which are absent.

#### 20 Conservation of the $\beta$ -GAP Sequences Across Species

To demonstrate that the  $\beta$ -GAP sequences are conserved, and that various species, including human, contain multiple copies of these genes a "Zoo blot" of various species of genomic DNAs was digested with EcoRI and analyzed by Southern blotting using the murine  $\beta$ -GAP6 BB500 probe. Under low stringency the blot showed detection of a multiplicity of bands in DNA from human,

rat, mouse, dog, rabbit and monkey. This indicates that multiple copies of the  $\beta$ -GAP sequences were found in many species including human. In addition, the conservation of the  $\beta$ -GAP multigene family predates speciation of murine and human and therefore is not the product of a recent gene duplication or rearrangement. The demonstration of interspecies sequence homology is significant because, in general, exons and regulatory regions tend to be conserved. Additional experiments show sequence homology for  $\beta$ -GAP in rabbits, mice, chickens and humans. Thus, the pattern of specific regions of retained homology suggests that the  $\beta$ -GAP sequences are retained by selective pressure.

Homology Between The  $\beta$ -GAP Sequences and the Promoters for  $\beta$ -globin

Sequences within all six of the 500 bp  $\beta$ -GAP clones show striking sequence and positional homology to mouse, rabbit and human  $\beta$ -globin promoter regulatory elements. Important regulatory elements within a 106 bp region of the  $\beta$ -globin promoter have been characterized (Myers et al., Science 232:613-618 (1986); Stuve and Myers, Mol. Cell Biol. 6:3350-3358 (1990)). Using saturation mutagenesis and 5' deletion promoters, Myers and his colleagues constructed a series of mutants that were used to identify four regulatory sequences. The four regulatory motifs were located between positions -95 and -26 which contain a CACCC element (positions -95 to -87), CCAAT and TATA box motifs at positions -79 to -72 and -30 to -26, respectively, and a 11bp repeat element

located between the CCAAT and TATA boxes (positions -53 to -32) that contains 2 imperfect duplicated repetitive elements (BDRE). The fact that these BDRE are essential for the expression of globin genes has been shown by  
5 deletional studies.

Comparison of the six 500 bp  $\beta$ -GAP sequences with the  $\beta$ -globin promoter sequences from various species showed several striking sequence homologies to  $\beta$ -globin regulatory elements. Analysis of the  $\beta$ -GAP sequence in  
10 this region revealed 5 regulatory motifs found in the  $\beta$ -globin promoters. These include the 5' CACCC erythroid element between positions -127 to -123, CCAAT and TATA box motifs between positions -109 to -105 and -30 to -26, respectively, the cap consensus sequence positions -13 to  
15 -10, and a fifth and more complex regulatory element involved a  $\beta$ -globin BDRE of a 10 and 11 bp sequence (base pair numbering was determined from sequence alignments with gaps inserted and does not reflect the true base pair position from the transcriptional start site).

20 In all the  $\beta$ -GAP clones, two of the four BDRE regulatory motifs were flanked by the CCAAT and TATA elements between positions -54 and -32, while two other BDREs were found immediately upstream of the TATA box (positions -11 to +1 and +3 to +12). All of these BDRE  
25 were conserved in sequence, and moreover, two of them were conserved in position (-54 and -32). It is significant that the BDREs conserved in  $\beta$ -GAP were conserved in globins from multiple species (mouse, rabbit, chicken and human) covering more than 100 million

years of evolution. This observation of evolutionary conservation indicates the  $\beta$ -GAP genes are old genes.

A final putative regulatory motif from the  $\beta$ -GAP clones was AGATAA (nucleotides -82 to -77), which  
5 is identical to the DNA consensus sequence for the transcriptional binding factors NF-E1. This family of DNA binding proteins (NF-E1a, b, and c) are involved in the erythroid and/or T-cell specific expression of many genes, including mouse and chicken adult  $\beta$ -globin, the  
10 heme pathway enzyme uroporphobilinogen (PBG) deaminase, the T-cell receptor and the leukemia virus HTLV III.

A closer inspection of the regions of homology between the  $\beta$ -GAP and mouse  $\beta$ -globin promoters reveals several features: 1) 18 of 26 base pairs match at  
15 positions -35 to -10 encompassing the consensus TATA motif (Bucher, J. Mol. Biol. 212:563-578 (1990)); 2) a region encompassing the  $\beta$ -GAP CCAAT box, positions -113 to -109 contains the  $\beta$ -globin regulatory element CACCC which has been shown to be essential for the appropriate  
20 expression of  $\beta$ -globin in erythroid cells; 3) a perfect match of the CCAAT element exists at positions -109 to -105; 4) the fourth matching region encompasses a  $\beta$ DRE element, located between the CCAAT and TATA boxes at positions -64 to -45 (this region contains 16 of 19 bp  
25 matches with no gaps); and 5) a consensus cap site sequence as defined by Bucher (Bucher, *supra*, 1990) and a putative transcriptional start site is identified at nucleotides -13 to +1.

Several other putative regulatory sequences are apparent in the  $\beta$ -GAP promoter. Between positions -68 and -37 and beginning 5 nucleotides distal of the TATA element are 4 palindromes. The 5 base pair repeat TCAGA appears twice within 24 base pairs. These repeats flank and are found within a globin-like imperfect direct repeat element (positions -57 to -47). Two longer palindromes with imperfect dyad symmetry of 12 bp, and 15 bp, positions -67 to -56 and -51 to -37, respectively, contain smaller internal palindromes of 7 bp, CCTCAGG (-66 to -60) and 5 bp repeat, TCAGA (-46 to -42), respectively. This  $\beta$ -GAP 33 bp  $\beta$ DRE-like region combining the two large 12 and 15 bp imperfect palindromes, the  $\beta$ -globin imperfect direct repeat element and the two TCAGA palindromic repeats shows about 50% (16/33) nucleotide sequence homology to the mouse  $\beta$ -globin promoter.

Expression of Genes Immediately downstream from the  $\beta$ -GAP Sequences in the Gastrointestinal tract

The pattern of specific regions of retained homology between the  $\beta$ -globin regulatory motifs and  $\beta$ -GAP promoters suggests: 1) the sequences have diverged from a common ancestral gene; and 2) the preserved regions in the  $\beta$ -GAP sequences play a critical role in the regulation of expression of their respective genes. Furthermore, the homology to promoters for genes intimately involved in iron metabolism, the occurrence of erythroid specific regulatory sequences, and the close proximity of these genes to the human locus responsible

for hemochromatosis, indicates a role for the  $\beta$ -GAP genes in iron metabolism.

To demonstrate that the  $\beta$ -GAP promoters regulate downstream messages, it is imperative to show that the associated genes encode transcribable messages. Moreover, such messages should be expressed in tissues involved in iron absorption, i.e. the gastrointestinal tract, if they are to be involved in the pathogenesis of hemochromatosis.

Northern blotting was performed with poly A+ RNA from various organs including the gastrointestinal tract. The blot was developed using a probe derived from a  $\beta$ -GAP(Q2<sup>b</sup>) cosmid clone. Total cellular RNA was prepared by the TRIzol™ Reagent method according to the manufacturer's instruction (Gibco/BRL, Gaithersburg, MD). poly A+ or mRNA was purified by oligo dT cellulose chromatography (Stratagene, San Diego, CA). RNA was analyzed on formaldehyde-agarose gels and transferred to Zeta Bind membranes as previously described (Evans, et al., Proc. Natl. Acad. Sci. (USA) 81:5532-5536 (1984)). The cosmid clone containing a  $\beta$ -GAP sequence that was used for the probe was obtained from a  $\alpha$  library. The clone was digested with ApaLI and KpnI to yield a 10kb fragment. The fragment was partially digested with BamHI to yield a 2 kb fragment encompassing the  $\beta$ -GAP sequences and an 8kb fragment further downstream containing the coding sequences for a  $\beta$ -GAP gene.

Northern blotting with showed the presence of polydisperse messages produced in tissues from stomach, duodenum, jejunum, kidney of 4-5 kb and 8 kb in size. The kidney showed less polydispersity with only the 8 kb band predominating. These results indicate that  $\beta$ -GAP promoter and upstream  $\beta$ -GAP coding sequences are expressed in the gastrointestinal tract and are associated with members of a multigene family of which the 5 Kb message of the jejunum is most prominent. The fact that this probe also recognized a band in the kidney and stomach, suggested that related members of a  $\beta$ -GAP family can be functioning in other tissues.

The size and complexity of the  $\beta$ -GAP mRNA products detected by northern blotting is consistent with  $\beta$ -GAP genes coding for a family of large proteins. These characteristics are more like those of a mucin protein family rather than an ion transport family of molecules. The homology to  $\beta$ -globin promoters, the occurrence of erythroid specific regulatory sequences and close proximity of nonclassical class I and  $\beta$ -GAP genes to the locus responsible for hemochromatosis in humans, an inheritable disease of iron metabolism indicates a role for the  $\beta$ -GAP genes and the nonclassical class I genes in iron metabolism. With this information in hand and the facts disclosed herein that  $\beta_2$ M-knockout mice have an unusually high incidence of hepatomas led to the understanding that these mice have a metabolic and pathological condition similar to hemochromatosis.



Isolation of murine  $\beta$ -GAP cDNAs from a mouse jejunal library.

- A  $\beta$ -GAP cosmid probe is used to screen a mouse jejunal cDNA library (Strategene  $\alpha$  ZAP Express kit).
- 5 Northern blots are used to indicate that the messages recognized by the  $\beta$ -GAP probe are expressed in the tissues examined. Clones which give positive signals are isolated, characterized and purified. DNA purified from the selected is digested with EcoRI and subjected to
- 10 Southern blot analysis. The blot is probed with the 800 bp  $\beta$ -GAP probe to verify that they contain the  $\beta$ -GAP genes.

Cloning of the human  $\beta$ -Gap Genes

- A human genomic DNA library produced in scOS
- 15 cosmid vector is prepared as described previously for producing a mouse genomic library in scOS (Strategene, San Diego CA). The isolation of the human  $\beta$ -GAP genes from the human scOS cosmid library is performed by screening clones with a class I MHC probe. The probe is
- 20 generated from exons 4 and 5 of the HLA-A2 gene, which encodes the highly conserved  $\beta_2$ M binding domain and the transmembrane region. Selected clones containing class I sequences are detected, and the Cosmids from these clones are purified, cut with the restriction enzyme EcoRI, run
- 25 on a 0.7% agarose gel and blotted onto a charged nylon membrane. The blot is hybridized with the class I probe, striped and rehybridized with the 800 bp  $\beta$ -GAP probe.

EXAMPLE II $\beta_2$ M KNOCKOUT MICE DEVELOP IRON OVERLOAD SIMILAR TO  
HEMOCHROMATOSIS

This example provides a method to analyze iron  
5 deficiency in an animal model where an MHC-encoded  
leczyme function has been genetically deleted. In  
addition, these mice are useful for evaluating the in  
vivo utility of carbohydrate ligands on the treatment of  
hemochromatosis and various iron related diseases such as  
10 atherosclerosis, arthritis or cancer.

The data concerning iron overload in the  $\beta_2$ M  
knockout mice is contained in Rothenberg and Volland,  
1994. Histologic examination of tissues from 12-18 month  
old knockout mice, contained on a standard diet, revealed  
15 evidence of hepatic necrosis. Iron stains of the tissues  
revealed iron deposition in the liver of all animals, and  
in the kidney, or the lung of approximately 10% of the  
animals. The standard diet contains 350mg/kg Ferric  
carbonate. When animals were placed on a "breeder diet",  
20 which contains in addition to ferric carbonate, 10mg/kg  
ferrous sulfate, iron stores rose dramatically. Iron  
deposition in the animals was also age related with the  
highest levels of iron seen in the oldest animals.  
Together these data indicate that the  $\beta_2$ M-knockout mice  
25 develop iron overload that is diet and age related. In  
addition we have shown that the animals develop hepatomas  
and others have reported that older animals develop  
diabetes (Faustman et al., Science 254: 1756-1761

(1991)). This constellation of pathologies mirrors human hemochromatosis.

Although the invention has been described with  
5 reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims.

## I Claim:

1. A composition, comprising a substantially purified carbohydrate ligand that specifically binds to a leczyne.
- 5 2. The composition of claim 1, further comprising a polypeptide or lipid moiety bonded to said carbohydrate ligand.
3. A composition, comprising a substantially purified carbohydrate ligand, wherein said carbohydrate  
10 ligand is an oligosaccharide.
4. A composition, comprising a substantially purified carbohydrate ligand, wherein said carbohydrate ligand is a polysaccharide.
5. A method of identifying a carbohydrate ligand  
15 that binds to a leczyne, comprising the steps of:
  - a. contacting a sample containing a carbohydrate ligand with a leczyne suspected to bind such a ligand; and
  - b. detecting whether binding of the ligand  
20 and the leczyne has occurred.
6. The method of claim 5, wherein said leczyne is an MHC-derived gene product.

7. The method of claim 6, wherein said MHC-derived gene product is a class I molecule.
8. The method of claim 5, wherein said MHC-derived gene product is a class II molecule.
- 5 9. The method of claim 5, wherein said MHC-derived gene product is an nonclassical class I molecule.
10. The method of claim 5, wherein said leczyne is a non-MHC derived gene product.
11. The method of claim 5, wherein said  
10 carbohydrate ligand is bonded to a polypeptide or lipid moiety.
12. The method of claim 5, wherein said carbohydrate ligand or said leczyne contains a detectable label.
- 15 13. The method of claim 5, wherein said carbohydrate ligand or said leczyne is associated with a cell.

14. A method of identifying a leczyne that binds to a carbohydrate ligand, comprising the steps of:

a. contacting a sample containing a leczyne with a carbohydrate ligand suspected to bind such a  
5 leczyne; and

b. detecting whether binding of the leczyne and the ligand has occurred.

15. The method of claim 14, wherein said leczyne is an MHC-derived gene product.

10 16. The method of claim 14, wherein said leczyne is a non-MHC derived gene product.

17. The method of claim 14, wherein said carbohydrate ligand is bonded to a polypeptide or lipid moiety.

15 18. The method of claim 14, wherein said carbohydrate ligand or said leczyne contains a detectable label.

19. The method of claim 14, wherein said carbohydrate ligand or said leczyne is associated with a  
20 cell.

20. A method of purifying a carbohydrate ligand that specifically binds to a binder, comprising the steps of:

- a. contacting a sample containing a  
5 carbohydrate ligand with a binder capable of binding such a ligand to form a ligand-binder complex;
  - b. separating the complex from the rest of the sample; and
  - c. dissociating the ligand from the complex  
10 to obtain the purified ligand.
21. The method of claim 20, wherein said binder is a leczyne.
22. The method of claim 20, wherein said binder is an antibody.
- 15 23. The method of claim 20, wherein said leczyne is an MHC-derived gene product.
24. The method of claim 20, wherein said leczyne is a non-MHC derived gene product.
25. The method of claim 20, wherein said  
20 carbohydrate ligand is bonded to a polypeptide or lipid moiety.

26. A method of purifying a leczyne that specifically binds to a carbohydrate ligand, comprising the steps of:

- a. contacting a sample containing a leczyne  
5 with a carbohydrate ligand capable of binding such a leczyne to form a ligand-leczyne complex;
  - b. separating the complex from the rest of the sample; and
  - c. dissociating the leczyne from the complex  
10 to obtain the purified leczyne.
27. The method of claim 26, wherein said leczyne is an MHC-derived gene product.
28. The method of claim 26, wherein said leczyne is a non-MHC derived gene product.
- 15 29. The method of claim 26, wherein said carbohydrate ligand is bonded to a polypeptide or lipid moiety.



30. A method of identifying a carbohydrate ligand that modifies the function of a leczyne-expressing cell, comprising the steps of:

a. contacting a sample containing such a  
5 carbohydrate ligand with a leczyne-expressing cell; and

b. subsequently assaying the cell to determine its function.

31. The method of claim 30, wherein said leczyne is an MHC-derived gene product.

10 32. The method of claim 30, wherein said leczyne is a non-MHC derived gene product.

33. The method of claim 30, wherein said carbohydrate ligand is bonded to a polypeptide or lipid moiety.

15 34. A method of identifying a leczyne that modifies the function of a carbohydrate ligand-expressing cell, comprising the steps of:

a. contacting a sample containing such a  
leczyne with a carbohydrate ligand-expressing cell; and

20

b. subsequently assaying the cell to determine its function.

35. The method of claim 34, wherein said leczyyme is an MHC-derived gene product.
36. The method of claim 34, wherein said leczyyme is a non-MHC derived gene product.
- 5 37. A method of modifying the function of a leczyyme-expressing cell, comprising contacting the cell with a carbohydrate ligand that binds the leczyyme.
38. The method of claim 37, wherein said leczyyme is an MHC-derived gene product.
- 10 39. The method of claim 37, wherein said leczyyme is a non-MHC derived gene product.
40. The method of claim 37, wherein said oligosaccharide ligand is bonded to a polypeptide or lipid moiety.
- 15 41. A method of modifying the function of a carbohydrate ligand-expressing cell, comprising contacting the cell with a leczyyme that binds the ligand.
42. The method of claim 41, wherein said leczyyme is an MHC-derived gene product.
- 20 43. The method of claim 41, wherein said leczyyme is a non-MHC derived gene product.

44. A method of identifying a peptide that binds to a carbohydrate ligand binding site of a leczyne, comprising the steps of:

- a. contacting a leczyne and a carbohydrate  
5 ligand known to bind the leczyne with a test sample containing a peptide to be identified;
- b. determining the amount of oligosaccharide ligand bound to the leczyne after said reacting; and
- c. comparing the amount of oligosaccharide  
10 ligand bound in the test sample with the amount of oligosaccharide ligand bound in a control sample, wherein a decreased amount of oligosaccharide ligand bound in the test sample relative to the amount of oligosaccharide ligand bound in the control sample indicates binding of  
15 the peptide to the leczyne.

45. The method of claim 44, wherein said peptide and said leczyne are contacted prior to contacting said oligosaccharide ligand.

20 46. The method of claim 44, wherein said oligosaccharide ligand or said leczyne contains a detectable label.

47. The method of claim 44, wherein said leczyne or said oligosaccharide ligand is associated with a cell.

48. The method of claim 44, wherein said test sample contains a peptide library.

49. A method of modifying a cell to produce a carbohydrate ligand, comprising introducing an expression  
5 vector encoding a leczyne into the cell to obtain expression of the leczyne, wherein said expression results in production of the oligosaccharide ligand by the cell.

50. The method of claim 49, wherein said leczyne is  
10 an MHC-derived gene product.

51. The method of claim 49, wherein said leczyne is a non-MHC derived gene product.

52. A method for modulating an immune response in an individual to an antigen, comprising administering the  
15 oligosaccharide ligand of claim 1 and the antigen.

53. The method of claim 52, wherein said administering results in an increase in the immune response to the antigen.

54. The method of claim 52, wherein said  
20 administering results in a decrease in the immune response to the antigen.

55. The method of claim 52, wherein said administering further comprises an immune suppressing agent.

56. The method of claim 52, wherein said antigen and said oligosaccharide ligand are covalently bonded.
57. The method of claim 52, wherein an adjuvant is administered along with said antigen and said  
5 oligosaccharide ligand.
58. A method for treating a disease state involving a leczyne, comprising administering an effective amount of the oligosaccharide ligand of claim 1.
59. The method of claim 58, wherein said disease  
10 state is an MHC-linked disease.
60. The method of claim 58, wherein said MHC-linked disease is an autoimmune disease.
61. The method of claim 58, wherein said MHC-linked disease is hemochromatosis.
- 15 62. The method of claim 58, wherein said disease state is an infection.
63. The method of claim 58, wherein said disease state is transplantation rejection.
- 20 64. A method for treating a disease state involving a leczyne, comprising administering an effective amount of a leczyne having a similar binding specificity for a carbohydrate ligand as the leczyne involved in the disease state.

65. The method of claim 64, wherein said leczyne is an MHC-derived gene product.

66. The method of claim 64, wherein said leczyne is a non-MHC derived gene product.

5 67. The method of claim 64, wherein said disease state is an MHC-linked disease.

68. The method of claim 67, wherein said MHC-linked disease is an autoimmune disease.

69. The method of claim 64, wherein said MHC-linked  
10 disease is hemochromatosis.

70. The method of claim 64, wherein said disease state is an infection.

71. The method of claim 64, wherein said disease state is transplantation rejection.

15 72. A method for detecting a genetic predisposition for hemochromatosis, comprising detecting a mutation in the heavy chain of a class I MHC molecule that reduces the ability of said heavy chain to associate with  $\beta_2$  microglobulin.

20 73. The method of claim 72, wherein said mutation eliminates a signal for the addition of a phosphate group.

74. The method of claim 72, wherein said mutation eliminates the ability of a phosphate group in said heavy chain to be de-phosphorylated for diagnosing hemochromatosis resulting from a reduction in the ability  
5 of a heavy chain of an MHC class I molecule to bind  $\beta_2$  microglobulin, comprising the steps of:

- a. isolating a class I MHC heavy chain from an individual to be tested;
- b. contacting the heavy chain with  $\beta_2$   
10 microglobulin under conditions suitable for associating a class I MHC heavy chain with  $\beta_2$  microglobulin; and
- c. detecting the association of said heavy chain with said  $\beta_2$  microglobulin, wherein a reduced association of said heavy chain with  $\beta_2$  microglobulin  
15 compared to the association of a control heavy chain with  $\beta_2$  microglobulin is diagnostic for hemochromatosis.

75. A protein that has the ability to distinguish self from non-self through the complimentary interaction of the protein and a carbohydrate and is not an  
20 immunoglobulin molecule or a T cell receptor molecule.

76. A method for treating a disease in a patient characterized by abnormal absorption of a metal from the patient's gastrointestinal tract, said method comprising the administration of a therapeutic dose of a  
25 carbohydrate which specifically binds to a protein coded by a gene within the IgGSF.

77. The method of claim 76, wherein the metal is iron.

78. A method for treating an autoimmune disease in a patient comprising administering a therapeutic dose of  
5 a carbohydrate decoy, said decoy specifically binding to a protein coded by a gene within the IgGSF.



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/15913

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 127/29; 424/94.6, 184.1, 278.1; 435/7.4, 200, 240.2; 436/501; 514/23; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 127/29; 424/94.6, 184.1, 278.1; 435/7.4, 200, 240.2; 436/501; 514/23; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CA, INPADOC, WPIDS, MEDLINE search terms: leczyme, histocompatibility, enzyme, catalysis, transplantation antigen, oligosaccharide, saccharide, carbohydrate, lectin

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOCHEMISTRY, Volume 82, Number 3, issued September 1977, Iwata et al, "Membrane Receptors of Mouse Lymphocytes for Various Lectins", pages 661-669, see entire document.	1, 2, 5-9, 11, 14, 15, 17, 20, 21, 23, 25, 26, 27, 29-31, 33-35
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, Number 31, issued 05 November 1992, Nag et al, "N-Linked Oligosaccharides of Murine Major Histocompatibility Complex Class II Molecule", pages 22624-22629, see entire document.	75

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &*	document member of the same patent family
* (I)* document referring to an oral disclosure, use, exhibition or other means		
* J* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 MARCH 1996

Date of mailing of the international search report

21 MAR 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

STEPHEN WALSH

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US95/15913

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	EUROPEAN JOURNAL OF BIOCHEMISTRY, Volume 213, issued 1993, Bezouska et al, "Characterization of the high-affinity oligosaccharide-binding site of the 205-kDa porcine large granular lymphocyte lectin, a member of the leukocyte common antigen family", pages 1303-1313, see entire document.	1,3-5,10 ----- 6-9,11-29
Y	THE JOURNAL OF IMMUNOLOGY, Volume 151, Number 1, issued 01 July 1993, Thor et al, "Monoclonal Antibody that Distinguishes between a Phosphorylated, $\beta_2$ -Microglobulin-Associated, and a Free, Nonphosphorylated, Chain of MHC Class I", pages 211-224, see entire document.	72-74
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Volume 90, issued December 1993, Rothenberg et al, "Biotinylated diaminopyridine: An approach to tagging oligosaccharides and exploring their biology", pages 11939-11943, see entire document.	12,18
A	CELL, Volume 66, issued 20 September 1991, Stamenkovic et al, "The B Lymphocyte Adhesion Molecule CD22 Interacts with Leukocyte Common Antigen CD45RO on T Cells and $\alpha 2$ -6 Sialyltransferase, CD75, on B Cells", pages 1133-1144, see entire document.	1-78
Y	US, A, 4,970,296 (SAITO ET AL) 13 November 1990, see columns 13-14.	49-51

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/15913

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/15913

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C07K 14/74; C08B 37/00; C12N 5/08, 9/24; C12Q 1/48, 1/68; A61K 31/70, 38/46, 39/00; G01N 33/566

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 1-25, drawn to a carbohydrate that binds a leczyne, a method of identifying a carbohydrate that binds a leczyne, a method of identifying a leczyne that binds a carbohydrate, and a method of purifying a carbohydrate that binds a leczyne.

Group II, claims 26-29, drawn to a method of purifying a leczyne.

Group III, claims 30-33 and 37-40, drawn to a method of identifying a carbohydrate ligand that modifies the function of a cell, and a method of modifying a leczyne expressing cell.

Group IV, claims 34-36 and 41-43, drawn to a method of identifying a leczyne that modifies the function of a cell, and a method of modifying the function of a carbohydrate expressing cell.

Group V, claims 44-48, drawn to a method of identifying a peptide that binds to a carbohydrate ligand binding site of a leczyne.

Group VI, claims 49-51, drawn to a method of modifying a cell via recombinant expression.

Group VII, claims 52-63 and 76-78, drawn to a method for modulating an immune response and a method for treating a disease state, each method comprising administering an oligosaccharide.

Group VIII, claims 64-71, drawn to a method for treating a disease state comprising administering a leczyne.

Group IX, claims 72-74, drawn to a method for detecting a genetic predisposition.

Group X, claim 75, drawn to a protein that has the ability to distinguish self from non-self.

The inventions listed as Groups I-X do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The carbohydrate ligand composition of Group I and the protein of Group X do not share a special technical feature because they are materially different in structure and function. The process steps used in the methods of Groups I-IX are the special technical features of each method and distinguish each of these methods from the others. Accordingly, none of Groups I-X share the same or corresponding special technical features because they each include special technical features and steps that are not shared with any other group.